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Derivation of Functional Endothelial Cells from Human Vascular Smooth Muscle Cells through Reprogramming

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Derivation of Functional Endothelial Cells from Human Vascular Smooth Muscle Cells through Reprogramming

By

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A thesis submitted to King's College London for
the degree of Doctor of Philosophy

Cardiovascular Division

Faculty of Life Sciences & Medicine

King's College London

August 2015

In dedication to my parents

ABSTRACT

Endothelial damage and dysfunction are implicated in cardiovascular pathological changes and the development of vascular diseases. In view of the fact that the spontaneous endothelial cell (EC) regeneration is a slow and insufficient process, it would be of great significance to explore alternative cell sources capable of generating functional ECs to repair damaged endothelium. Indeed, recent achievements of cell reprogramming to convert somatic cells directly to other cell types provide new powerful approaches to study endothelial regeneration. Endothelial damage is often followed by smooth muscle cell (SMC) proliferation and accumulation. If we could arbitrarily convert SMC into EC, a novel cell regeneration strategy may be achieved. The aim of the present study is to test the hypothesis whether functional ECs could be derived from human vascular SMCs through reprogramming.

Based on a combined protocol of reprogramming and differentiation, human vascular SMCs were first reprogrammed for 4 days to achieve a partially converted state expressing vascular progenitor marker CD34, by introducing four transcription factors *OCT4*, *SOX2*, *KLF4* and *c-MYC*. Partially reprogrammed SMCs were then subjected to defined media and culture conditions to induce the differentiation towards an endothelial lineage. The differentiated cells expressed EC markers at the mRNA and protein level. Next, CD34-positive cells were selected from the heterogeneous population of vascular progenitors and further maintained in endothelial-promoting conditions. The CD34-positive cells were able to give rise to a more homogenous endothelial population with a repertoire of endothelial characteristics. These SMC-converted ECs displayed typical endothelial functional properties including Nitric Oxide (NO) production, acetylated-low density lipoprotein (LDL) uptake and angiogenic ability *in vitro* and *in vivo*. More importantly, these human SMC-derived ECs showed therapeutic angiogenic capacity that improved blood flow recovery when applied in a murine hindlimb ischaemia model as well as the ability to assemble into endothelium-like layer in tissue engineered vascular graft. Furthermore, the mechanisms involved in SMC to EC conversion were explored. Comprehensive analyses indicated that mesenchymal-to-

epithelial transition was required to initiate the conversion from SMCs into vascular progenitors. In addition, components of the Notch signalling pathway, hairy and enhancer of split 5 (HES5) and Jagged1 (JAG1), regulated the differentiation of vascular progenitors towards an endothelial lineage.

Together, we provide the first evidence of the conversion of human SMC towards functional endothelial lineage through mechanisms involving mesenchymal-to-epithelial transition and the Notch signalling pathway.

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ACKNOWLEDGEMENTS

First and foremost I would like to sincerely thank my supervisor Professor Qingbo Xu for his support and encouragement throughout my PhD experience. It has been an honour to work under his guidance. I truly appreciate all his contributions of time, ideas, and funding to make my PhD experience productive and stimulating.

I would like to thank Dr. Andriana Margariti for her daily supervision during the first two years of my PhD and her expertise, advice and support throughout the whole project. I want to thank Dr. Yanhua Hu for all the help and suggestions especially with animal experiments.

Many thanks to all the past and present members of the Xu lab for all kinds of help, advice and encouragement. I have learnt a lot from everyone. Thank them and other members of the Division for creating a friendly and cheerful working environment. Those chocolates/nuts/coffee/macarons/peanut butter pretzels sharing moments always made a long day go easier. In particular for this thesis preparation and writing, I want to thank Dr. Alexandra Le Bras for the inspiring discussions and invaluable suggestions. I would also like to thank Dr. Claire Potter and Dr. Ka Hou Lao for their help in reading the final version of my thesis.

I want to thank all my friends for their supports and being patient. Lily, thank you for the unconditional support and cheering me up everyday.

Most of all, I would like to express my deepest gratitude to my dear parents. Without their support, understanding, and most of all love, the completion of this work would not have been possible.

Thank you all. It has been a great journey.

DECLARATION

I, Xuechong Hong, confirm that the work presented in this thesis is my own and I have been involved in the design, planning and conduct of all the experiments and the thesis writing.

Expert assistance was provided in some aspects of the project by the following colleagues from the Cardiovascular Division of King's College London.

Dr. Yanhua Hu performed the *in vivo* part of the *in vivo* angiogenesis Matrigel plug assay and the mouse hindlimb ischaemia model.

Dr. Andriana Margariti generated the plasmid TetO empty vector, pCAG empty vector, and the plasmid pGL3-HES5 luciferase reporter vector.

ABBREVIATIONS

3D	3 Dimensional
Ac-LDL	Acetylated Low Density Lipoprotein
AKT	Protein Kinase B
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
c-Myc/c-MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
CO₂	Carbon Dioxide
Ct	Threshold Cycle
Cy3	Carbocyanine 3
DAPI	4', 6-Diamidino-2-Phenylindole
da-FA-OMe	Deaminated Fluorescein Amine Methyl Ester
DII/DLL	Delta-like Ligand
DMEM	Dubelco Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
ddH₂O	Double-distilled Water
EB	Embryoid Body
EC	Endothelial Cell
ECL	Enhanced Chemiluminescent
ECM	Extracellular Matrix
EDTA	Ethylene-Diaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGM-2	Endothelial Growth Medium-2

ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
ERK	Extracellular-Signal-Regulated Kinase
ESAM	Endothelial Cell Selective Adhesion Molecule
ESC	Embryonic Stem Cell
ESC-EC	Embryonic Stem Cell-derived Endothelial Cell
ETS	E-Twenty Six
ETV2	ETS Variant 2
F-12	Nutrient Mix F-12
FA-OMe	Fluorescein Amine Methyl Ester
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Flk1	Fetal Liver Kinase 1
FOX	Forkhead
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GO	Gene Ontology
H&E	Haematoxylin & Eosin
HEK	Human Embryonic Kidney
HES5	Hairy Enhancer-of-Splits 5
HEY	HES-related Protein
HUVEC	Human Umbilical Vein Endothelial Cell
ICM	Inner Cell Mass
Ig	Immunoglobulin
IgG	Immunoglobulin with G Class Heavy Chains
IL	Interleukin
iPS cell	Induced Pluripotent Stem Cell
iPS-EC	Induced Pluripotent Stem Cell-derived Endothelial Cell
JAG1	Jagged 1
JAMs	Junctional Adhesion Molecules
kDa	Kilodalton
KDR	Kinase insert Domain Receptor
Klf4/KLF4	Krüppel-like Family of Transcription Factor-4

KO	Knock-Out
KO-DMEM	Knockout Dubelcco's Modified Eagle Medium
LB	Luria Bertani
MAPK	Mitogen-activated Protein Kinases
MEF	Mouse Embryonic Fibroblast
MET	Mesenchymal to Epithelial Transition
miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
NaN₃	Sodium Azide
NEAA	Non-Essential Amino Acids
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ns	Not Significant
NT	Non-Targeting
SCID	Severe Combined Immunodeficiency
O₂	Oxygen
Oct4/OCT4	Octamer-binding Transcription Factor-4
ORF	Open Reading Form
PAD	Peripheral Artery Disease
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PiPS Cell	Partial-induced Pluripotent Stem Cell
RP-SMC	Partially Reprogrammed Smooth Muscle Cell
qPCR	Quantitative Polymerase Chain Reaction
RBP-J	Recombination Signal Binding Protein for Immunoglobulin kappa J
RIPA	Radio Immunoprecipitation Assay
RNA	Ribonucleic Acid
RNA-Seq	RNA-Sequencing

ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RPKM	Reads Per Kilobase per Million
RT	Reverse Transcription
RTKs	Receptor Tyrosine Kinases
Sca-1	Stem Cell Antigen-1
SEM	Standard Error Of The Mean
SDS	sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel
shRNA	Short Hairpin Ribonucleic Acid
SM22α	Smooth Muscle Protein 22-alpha
SMA	Smooth Muscle Actin
SmBM	Smooth Muscle Basal Medium
SMC	Smooth Muscle Cell
SMC-CD34-EC	CD34-Positive Partially Reprogrammed Smooth Muscle Cell-derived Endothelial Cell
SMC-EC	Smooth Muscle Cell derived Endothelial Cell
SMMHC	Smooth Muscle Myosin Heavy Chain
Sox2/SOX2	SRY (Sex-Determining Region Y)-related HMG (high-mobility-group)-Box protein-2
SRF	Serum Response Factor
SSEA-3/4	Stage-Specific Embryonic Antigen-3/4
TGF-β	Transforming Growth Factor β
TLR	Toll-Like Receptor
Tris	Tris(Hydroxymethyl) Aminomethane
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TU	Transducing Unit
UASMC	Umbilical Artery Smooth Muscle Cell
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VSV-G	Vesicular Stomatitis Virus G Protein
VWF	Von Willebrand Factor

CHAPTER 1. INTRODUCTION

1.1 The Vascular System

1.1.1 Human Blood Vessel Structure and Function

Human blood vessels compose the indispensable parts of the circulatory system to transport blood throughout the whole body. Blood vessels can be mainly classified into arteries, veins and capillaries based on the anatomic structures and function. Arteries deliver the blood carrying oxygen and nutrients from the heart to different organs and tissues, whereas capillaries allow the exchange of nutrients with metabolic wastes between the blood and the tissues. Veins function by carrying the blood back to the heart. Blood vessels generally consist of three layers: tunica intima, tunica media and tunica adventitia (Figure 1.1):

Tunica intima is composed of endothelium, sub-endothelial layer and internal elastic lamina. An intact monolayer of endothelial cells (ECs) forms the endothelium and serves as a selectively permeable barrier between the blood and vessel wall. Sub-endothelial layer is constructed by connective tissue and branch cells. Internal elastic lamina consists of a network of elastic fibres and has a role in the maintenance of blood pressure (McKinley and O'Loughlin 2006).

Tunica media is a thick middle layer of smooth muscle cells (SMCs) and complex extracellular matrix. Due to the contractile ability of SMCs, tunica media provides support to the vessel and regulates blood flow and blood pressure via controlling the vessel diameter.

Tunica adventitia is the outermost layer and is primarily composed of connective tissue and matrix-secreting fibroblasts. It provides structural support to the vessel and helps it to fit into the surrounding structure. Recent evidence indicates that the tunica adventitia also serves as an important vascular stem/progenitor cells reservoir (Hu and Xu 2011).

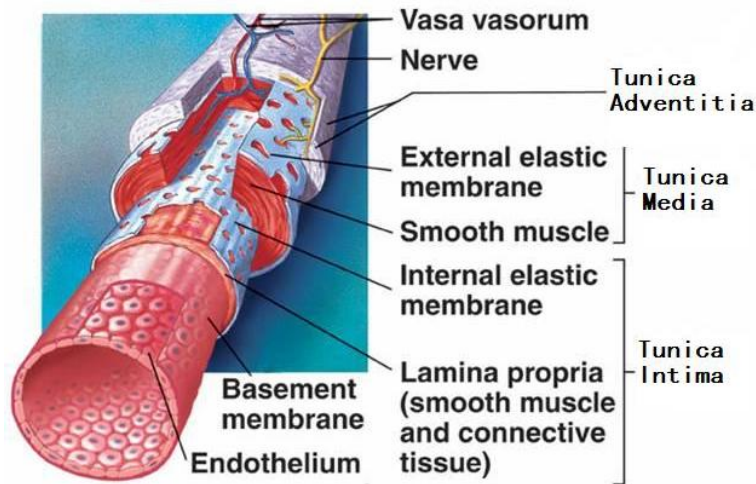


Figure 1.1 Basic structure of human vessels

Human vessels generally consist of three main layers: tunica intima, tunica media and tunica adventitia. ECs array the most inner layer of the vessels. SMCs predominantly contribute to the tunica media to support the vascular structure and to control the blood flow by contractility. Figure is adapted from (McKinley and O'Loughlin 2006).

1.1.2 Vascular Endothelial Cell

Vascular ECs array the most inner layer of the entire circulatory system, from the largest arteries and veins to the smallest capillaries, which serve as a semi-selective and non-adherent interface between blood and the underlying tissues. In response to diverse physiological and pathological stimuli, ECs are actively involved in regulating endothelium permeability, modulating vascular tone, regulating blood coagulation, and many other biological processes. EC dysfunctions precede the development of many vascular pathological conditions such as atherosclerosis, hypertension, and diabetes (Deanfield, Halcox et al. 2007).

Under *in vitro* culture conditions, confluent ECs have the typical “cobblestone-like” morphology with slow rates of growth and motility. In contrary, sparse ECs exhibit spindle or flat shapes, are actively motile and are sensitive to growth factor stimulation (Aird 2007). There is no exclusive marker that is solely restricted to EC. Generally, a cluster of markers are recognised to identify EC including platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial-cadherin

(VE-Cadherin), Claudin 5, endothelial nitric oxide synthase (eNOS), von-Willebrand factor (VWF), vascular endothelial growth factor (VEGF) receptors, etc. *In vitro*, the physiological function of ECs can be assessed by a series of tests such as the uptake of low-density lipoprotein, angiogenic ability, synthesis and release of nitric oxide (NO), regulation of permeability, inflammatory activation response to TNF- α and production of appropriate extracellular matrix to form basal lamina (Glaser, Gower et al. 2011).

The physiological functions of EC *in vivo* are briefly summarised as follows (Figure 1.2):

Endothelial Cell Barrier

The integrity of the endothelium is the foundation of vascular homeostasis. The cell-cell junctional structures, which link EC with each other to form a continuous monolayer, profoundly contribute to the regulation of permeability and the maintenance of the endothelium integrity. Furthermore, EC junctions actively participate in transferring intercellular signals between adjacent cells. EC junctions are formed by transmembrane adhesive proteins that link to specific cytoplasmic and cytoskeletal molecules. Based on the molecular structure and functional characterisation, EC junctions can be mainly classified into tight junctions and adherens junctions (Dejana 2004).

Tight junctions serve as the physical barrier and control the permeability of ions and water-soluble solutes through the paracellular gaps. Tight junctions of EC consist of claudin family, occludin, junctional adhesion molecules (JAMs) and endothelial cell selective adhesion molecule (ESAM), among which Claudin 5 is an EC-specific transmembrane adhesion protein (Morita, Sasaki et al. 1999). Claudin 5-deficient mice died within 10 hours after birth due to the defect in the blood-brain barrier (Nitta, Hata et al. 2003).

Adherens junctions among ECs mainly refer to VE-Cadherin, which is a calcium dependent transmembrane receptor and is one of the most representative markers for ECs. VE-Cadherin plays a key role in modulating endothelium activities by interacting with a variety of intracellular components which trigger complex signalling networks including reorganising the cytoskeleton and regulating gene

transcription. VE-Cadherin deficient embryos exhibit extensive endothelium remodelling and maturation failures that lead to lethality at day 9.5 of gestation (Carmeliet, Lampugnani et al. 1999). The cytoplasmic domain of VE-Cadherin interacts with β -catenin, plakoglobin and p120 to transmit signalling downstream (Vestweber 2008).

PECAM-1 is another important EC intercellular junction protein belonging to the immunoglobulin super-family. In addition to its adhesive property, PECAM-1 also functions as a scaffold for signalling and adaptor molecules (Ilan and Madri 2003). PECAM-1 mediates the transduction of multiple signalling pathways mainly through the phosphorylation of the specific tyrosine residues within the cytoplasmic tail (Newman and Newman 2003).

Vascular Tone Regulation

Endothelium regulates vascular tone through secreting EC-derived vasodilators including NO, prostacyclin and other factors. These EC-derived factors mediate the relaxation of SMCs to achieve vasodilation. NO is recognised as the primary factor for vasodilation which is synthesised in ECs by eNOS and that are dependent on its cofactors including free calcium (Ca^{2+}) and L-arginine (Michel and Vanhoutte 2010). Soluble guanylate cyclase (sGC) within SMCs can be activated by EC-released NO to dephosphorylate GTP to cGMP, which serves as a second messenger for signalling SMC relaxation (Carvajal, Germain et al. 2000). In addition to the modulation of vascular tone, NO also has other vessel-protective roles including regulating the growth of local cells, and inhibiting the aggregation and adhesion of inflammatory cells and platelets to endothelial surface (Tousoulis, Kampoli et al. 2012).

Regulation of Blood Coagulation and Platelet Aggregation

Healthy ECs possess anticoagulant and antithrombotic functions to keep the vascular patency. Blood coagulation is prevented by ECs through synthesising and displaying inhibitors for tissue factor pathway and thrombins. ECs also express molecules for protein C activation which can demolish certain clotting factors and inhibits coagulation (Poher and Sessa 2007). ECs can physically separate the interaction of platelets and collagen which can activates platelets. ECs also secrete anti-platelet

molecules like prostacyclin, NO and prostaglandin- E_2 to inhibit the adhesion and activation of platelet on endothelial surface (van Hinsbergh 2012). Under physiological condition, endothelium prevents the inflammatory cells adhesion by failing to express adhesion molecules that mediate leukocytes attachment.

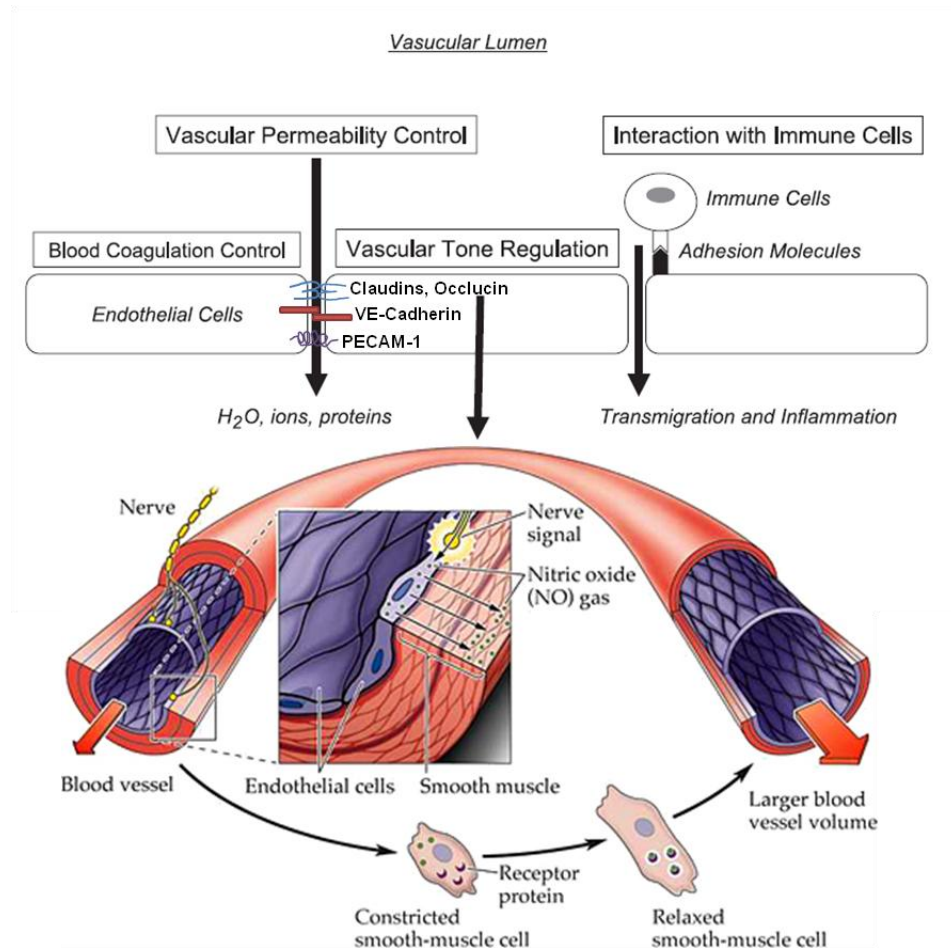


Figure 1.2 Physiological functions of EC

The intact endothelium form by functional ECs plays key role in maintaining vascular homeostasis. Endothelium is the frontline barrier to control vascular permeability, blood coagulation, platelet and inflammatory cells adhesion and aggregation. Functional endothelium regulates vascular tone through secreting vasodilators to react on the SMCs underneath. Figure is adapted from (Hirase and Node 2012).

1.1.3 Vascular Smooth Muscle Cell

Vascular smooth muscle cells (SMCs) and their products extracellular matrix (ECM) compose the majority of the vascular wall. The thick layer of SMCs and surrounding ECM serve to maintain the structural integrity of blood vessels from the high pressure generated from circulating blood. The contraction and relaxation of vascular SMCs coordinate the vascular tone and vessel lumen diameter that are important in the regulation of blood pressure and blood flow distribution (Rensen, Doevendans et al. 2007). Normal adults vascular SMCs express a repertoire of contractile proteins, ion channels, calcium handling proteins and cell surface receptors (Metz, Patterson et al. 2012).

One crucial feature of SMCs is that they retain phenotypic plasticity to switch between a quiescent, contractile phenotype and a proliferative, synthetic phenotype. Under normal physiological condition, SMCs maintain contractile phenotype with extremely low proliferation rate and synthetic activity. In response to environmental stimuli and extracellular signals, SMCs lose contractile proteins and shift to the synthetic phenotype with higher proliferative and migratory capacity and secrete high levels of ECM components such as collagen, elastin and proteoglycans (Owens, Kumar et al. 2004, Beamish, He et al. 2010). Morphologically, contractile SMCs exhibit elongated, spindle-like shape whereas synthetic SMCs (also referred as epitheloid SMCs) display a less elongated, more cobblestone-like morphology (Hao, Gabbiani et al. 2003). The expression patterns of protein markers have been widely adopted to characterise the different phenotypic states of SMCs. Contractile SMCs display a profile of contractile proteins including α -smooth muscle actin (α -SMA), Calponin, SM22 α , smooth muscle myosin heavy chain (SM-MHC). In contrary, synthetic SMCs contain minimal contractile proteins but large amounts of Golgi apparatus, rough endoplasmic reticulum and ribosomes related proteins (Beamish, He et al. 2010). The phenotypic modulation of SMCs is actively involved in different pathologic situations including atherosclerosis, restenosis, asthma, hypertension and cancer.

A complex transcriptional network mediates the phenotypic switch of SMCs. The promoter/intronic sequences of most SMC signature genes contain one or more conserved CArG elements, which interact with transcription factor serum response

factor (SRF) homodimers and coactivator Myocardin. Many other factors can promote or repress the expression of SMC genes through regulating the SRF/Myocardin-CArG interactions (Alexander and Owens 2012). For example, Krüppel-like factor 4 (KLF4), a member of the zinc finger-containing transcription factor family, can strongly inhibit Myocardin-SRF-CArG that leads to the suppression of SMC marker expression such as α -SMA, SM22 α , and SM-MHC (Liu, Sinha et al. 2005). In addition, KLF4 can also suppress SMC marker expression through binding to the transforming growth factor (TGF)- β response element located within their promoter region (Liu, Sinha et al. 2003). Interestingly, a recent study showed that KLF4 initiate the phenotypic switch of SMCs to macrophage-like cells within atherosclerotic lesions, through binding to and inhibiting multiple SMC marker-encoding genes (Shankman, Gomez et al. 2015).

1.1.4 Vascular System Development

1.1.4.1 Embryonic Vascular Development

The formation of blood vessels during embryogenesis is usually through two types of biological processes: vasculogenesis and angiogenesis. Vasculogenesis is the primary process to form blood vessel *de novo* by the differentiation of mesoderm-derived endothelial progenitors (angioblasts), while angiogenesis is the sprouting of the new blood vessels from the pre-existing vascular networks. Many events that occurring during embryonic vascular development are recapitulated under situations of neoangiogenesis in the adult (Coultas, Chawengsaksophak et al. 2005).

Embryonic vasculogenesis occurs simultaneously with the differentiation and specification of ECs under the control of a spatiotemporally precise signalling network. During the gastrulation stage of mammalian embryogenesis, a VEGFR2-positive mesoderm precursor population emerges in the posterior primitive streak in response to basic fibroblast growth factor (bFGF) and bone morphogenetic protein (BMP) signals. Next, these VEGFR2-positive angioblasts migrate to both the extra-embryonic yolk sac and the embryo to independently differentiate into ECs and form vascular structures. Angioblasts migrate towards the extraembryonic yolk sac to

form the blood islands. The peripheral cells of the blood islands give rise to ECs, whereas the central cells become haematopoietic progenitors. The blood islands further remodel and form a primary vascular plexus. The angioblasts migrated into the embryo aggregate together and form the dorsal aorta, cardinal veins and vascular plexus with the specification of ECs into arterial or venous fates (Coultas, Chawengsaksophak et al. 2005).

A variety of transcription factors have fundamental roles in regulating endothelial specification and differentiation including members of the E-twenty six (ETS), SOX, GATA, Forkhead (FOX), Krüppel-like families (KLF) (De Val and Black 2009). Different sets of transcription factors orchestrate distinct stages in EC development. ETS transcription factors can potentially regulate almost all typical endothelial genes by interacting with the ETS binding sites contained in their promoter regions (Dejana, Taddei et al. 2007). The transient activation of the most important ETS factor *ETV2* within a specific development window is requisite to guide the VEGFR2-positive precursor cells to differentiate towards EC lineage (Kataoka, Hayashi et al. 2011). KLF factors *KLF2* and *KLF4* are closely associated with EC development and maturation. The promoter regions of many endothelial genes including VEGFR2, eNOS, and thrombomodulin contain KLF binding sites (Atkins and Jain 2007).

Angiogenesis is the formation of new capillaries from the pre-established vasculature, which contributes to the remodelling of the primary vascular plexus to form a functional mature circulatory network. The basic angiogenic steps include growth factors induced ECs activation, breakdown of vascular basement membranes, ECs migration and proliferation, and subsequent stabilisation and maturation of blood vessels (Folkman 2003). At the onset of angiogenesis, ECs are activated in response to the stimulation of proangiogenic factors such as VEGF, epidermal growth factor (EGF), fibroblast growth factor (FGF) and a number of other factors. Activated ECs start to proliferate and release proteolytic enzymes to dissolve the basement membrane. At the same time, ECs are specified into tip or stalk cells due to the cross-talk between VEGF and the Notch signalling pathway. Endothelial tip cells migrate toward the angiogenic stimulus and guide the direction for the proliferating endothelial stalk cells to form tubular structures. Stalk cells connect to each other through junctional proteins and produce basement membrane components to enforce the integrity of the sprout (Phng and Gerhardt 2009, Potente, Gerhardt et al. 2011).

Finally, the remodelling and maturation of vascular structures require dynamic interactions between ECs with neighbouring cells and basement membrane (Eilken and Adams 2010).

Vascular SMCs arise from multiple independent origins during embryonic development. These include neural crest, second heart field, somites, splanchnic mesoderm, mesothelium, mesoangioblasts and various progenitor/stem cells (Majesky 2007). During vasculogenesis and angiogenesis, SMCs and pericytes are recruited by ECs from surrounding mesenchyme following the generation of primary vessels to form a mature vascular system. SMCs developed from different sources compose the whole circulatory system in a complex mosaic pattern. The versatility of SMCs developmental origins influences the pathogenesis of vascular disease in a site-specific way (Sinha, Iyer et al. 2014). Transforming growth factor β (TGF- β) signalling plays an important role in stimulating SMCs maturation, proliferation, migration and producing extracellular matrix (Pardali, Goumans et al. 2010). Deficiency of TGF- β receptors in mice cause abnormal mural cells development that leads to a fragile vascular structure and impaired integrity (Goumans and Mummery 2000).

1.1.4.2 Embryonic Stem Cell as a model to study vascular development

Due to the ethical issues, most of our knowledge of *in vivo* vascular development during mammalian embryogenesis is based on mouse models. The discovery of embryonic stem cell (ESC) has made a breakthrough advance in the field of developmental biology. ESCs are pluripotent cells isolated from the inner cell mass of blastocyst that represent a revolutionary *ex vivo* tool to study the development of different system, especially for human. ESCs automatically aggregate into three-dimensional (3D) embryoid body (EB) in culture, which can recapture the differentiation of the three germ layers during embryogenesis: ectoderm, mesoderm and endoderm. Using mouse EB formation to study vascular development, the expression of VEGFR2 appears at day 3 followed by the expression of PECAM-1, Tie1, Tie2, and VE-Cadherin on days 4 and 5 (Vittet, Prandini et al. 1996). Human EB shows different vascular development pattern than mouse. VEGFR2 and Tie2 are

already expressed at certain level in undifferentiated human ESCs. During the spontaneous differentiation of human EB, the level of a group of endothelial related markers including CD34, PECAM-1, and VE-Cadherin is gradually turned on and reach a maximum at day 13-15 (Levenberg, Golub et al. 2002). The extensive formation of vasculature-like structures can also be observed within the human EB. Interestingly, a study showed that CD34-positive cells isolated from human EBs at day 10 can be selectively induced to either endothelial-like or smooth muscle-like cells in response to different culture conditions (Ferreira, Gerecht et al. 2007).

Many important developmental concepts have been tested using ESC model. For example, the concept of haemangioblast serving as the common precursor of haematopoietic and endothelial cells during development was first proved using the mouse EB model and then in human EB model. A VEGFR2-positive colony-forming precursor population is identified in the EB that can be further differentiated into both haematopoietic and endothelial lineage with the stimulation of VEGF (Choi, Kennedy et al. 1998, Kennedy, D'Souza et al. 2007). However, this VEGFR2-positive population could also give rise to smooth muscle-like cells or cardiomyocytes with different stimulation, which indicated that the VEGFR2-positive cells may represent a broader mesodermal progenitor (Yamashita, Itoh et al. 2000, Schroeder, Fraser et al. 2003).

1.1.4.3 Evidence of ECs and SMCs may share common progenitor during development

Tracing back to the embryonic origins, both ECs and SMCs are mainly derived from the same location: mesoderm layer. ECs are derived from mesodermal angioblasts during embryogenesis. A large portion of SMCs are developed from multiple mesodermal origins including lateral plate mesoderm, splanchnic mesoderm and somatic or paraxial mesoderm (Sinha, Iyer et al. 2014). For example, descending aorta that is originated from dorsal aorta in the embryo for example, ECs and SMCs that form the dorsal aorta are both first derived from splanchnic lateral plate mesoderm, and then replaced by ECs and SMCs derived from somite-mesoderm (Wasteson, Johansson et al. 2008, Sato 2013). After grafting quail somites into chick

embryos, the contribution of somite-derived cells to both aortic ECs and SMCs was observed (Pouget, Gautier et al. 2006).

An important study using mouse embryo supported the idea of common progenitor during development. Branchyury- and VEGFR2-positive colony-forming cells were isolated from embryonic day (E) 7.9-7.5 mouse embryo mesoderm area. These cells could give rise to the colony containing cells expressing markers of the endothelium and smooth muscle when cultured *in vitro* (Huber, Kouskoff et al. 2004). A variety of signals including bFGF, BMP, Wnt, and Notch have been implicated in mesoderm development. The extensive interaction with the establishment of precisely spatiotemporal concentration gradients of these signals lead to specific mesodermal patterning in the embryo (Sinha, Iyer et al. 2014). During chick embryogenesis, the cross-talks between Notch, Wnt and BMP control the differentiation potential of ventral mesoderm-derived precursors towards ECs or SMCs (Shin, Nagai et al. 2009).

Using ESC to vascular lineage differentiation as a model, the existence of common progenitors for ECs and SMCs have been demonstrated by many studies. Yamashita *et al.* first claimed that VEGFR2-positive cells from mouse ESCs could give rise to both ECs and SMCs in response to VEGF or PDGF-BB stimulation respectively (Yamashita, Itoh et al. 2000). Subsequently, Ferreira *et al.* isolated the CD34-positive vascular progenitor cells during human EB growth which could differentiate into both endothelial and smooth muscle-like cells (Ferreira, Gerecht et al. 2007). The CD34-positive vascular progenitor cells with the EC and SMC differentiation capacity have also been identified in a 2D human ESCs culture system (Hill, Obrtlíkova et al. 2010) (Figure 1.3).

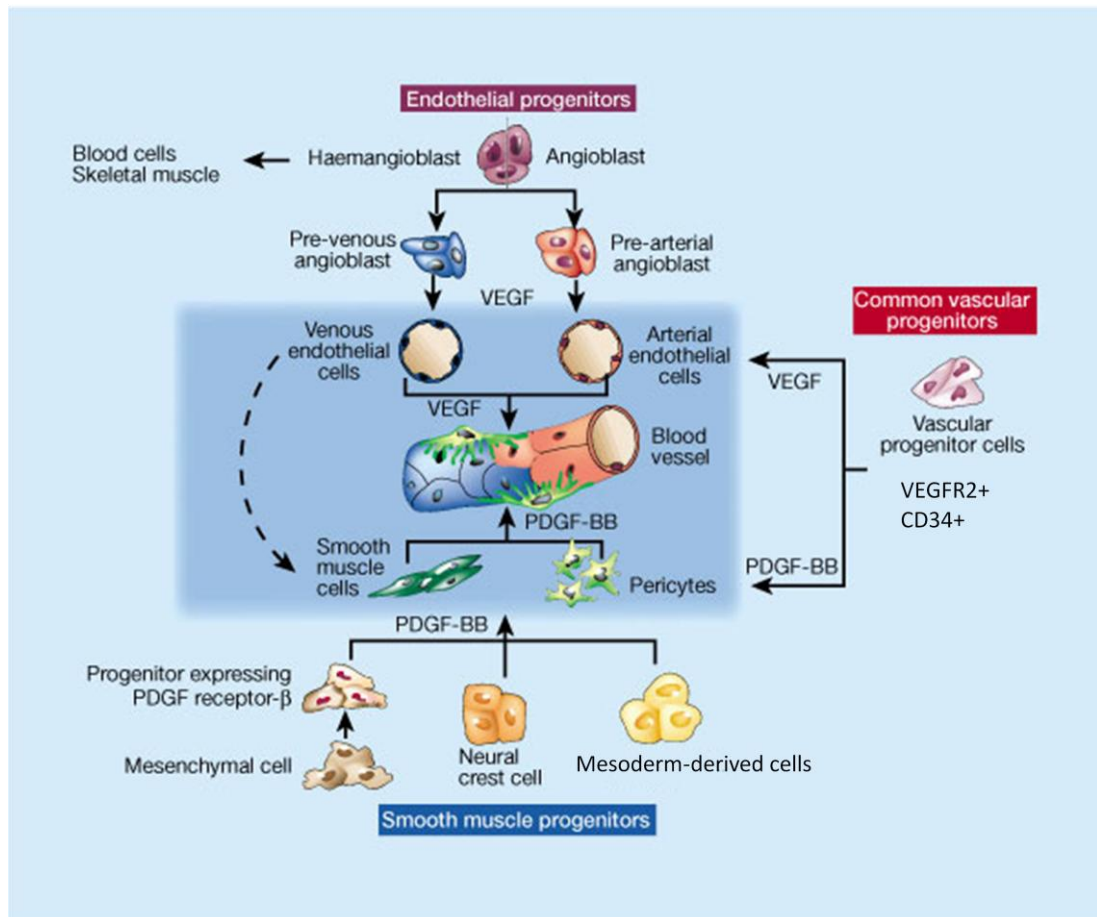


Figure 1.3 The development of ECs and SMCs and the possible existence of common vascular progenitors

ECs are developed from mesoderm-derived angioblasts and differentiate towards arterial or venous fate along with vasculogenesis. SMCs are derived from multiple origins including mesoderm. SMCs are recruited by ECs for vascular maturation. The existence of common vascular progenitors for both EC and SMC is supported by studies using ESCs as the model for vascular development. Figure is adapted from (Carmeliet 2000)

1.1.4.4 The Notch signalling pathway and vascular development

The Notch signalling pathway extensively participates in diverse vascular cell activities during embryonic and postnatal development. The Notch pathway is a highly conserved cell-cell signalling system consisting of transmembrane ligands (Jag1, Jag2, Dll1, Dll3, and Dll4), transmembrane receptors (Notch1-4), intracellular signal transducer and effectors. For canonical Notch signalling pathway, the receptors on the signal-receiving cells are activated upon ligands binding and the

intracellular domains of the receptors are proteolytic cleaved and released. The Notch intracellular domain then translocates into the nucleus and interacts with DNA-binding proteins recombination signal binding protein for immunoglobulin kappa J (RBP-J) to activate downstream targets genes such as Hairy enhancer-of-splits (HES) and HES-related proteins (HEY) family (Bray 2006) (Figure 1.4). The Notch signalling is inhibited when the receptor is interacting with the ligand presented within the same cell, which is known as the *cis*-inhibition of Notch (del Alamo, Rouault et al. 2011). The *cis*-inhibition of Notch regulates the ability of a cell to receive the signal from the adjacent cells. In addition, the Notch pathway can also function through non-canonical mechanism by interacting with Wnt signalling in a ligand/transcription-independent way (Andersen, Uosaki et al. 2012).

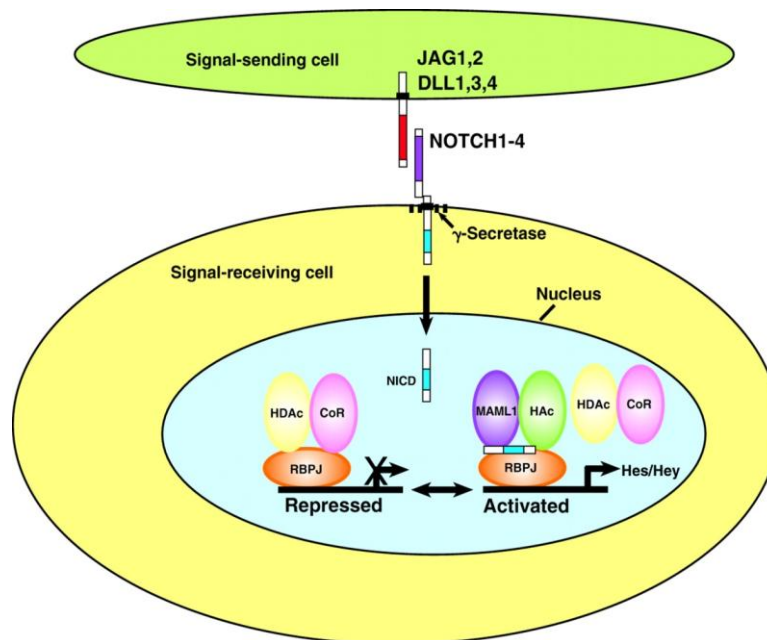


Figure 1.4 The Notch signalling pathway

The canonical Notch pathway is a ligand-receptor signalling system. Signal-sending cells express ligands to bind to the receptors on the signal-receiving cells. The intracellular domain of the receptors are proteolytic cleaved and translocate into the nucleus to regulate downstream target genes. Figure is adapted from (Gridley 2007).

The Notch ligands and receptors are widely expressed by vascular cells and the delicate spatial and temporal regulation of the Notch pathway control various

vascular processes including ECs and SMCs differentiation, angiogenesis, arterial-venous specification. Loss-of-function studies showed that the null mutation of either receptor Notch1, Notch2 or ligand Jag1, Dll1, Dll4 all causes severe vascular defects and embryonic lethality (Gridley 2007).

The Notch pathway actively controls multiple aspects of EC differentiation and specification. Using avian embryos, Sato *et al.* showed that Notch signalling guided the migration of the angioblasts within somites mesoderm towards dorsal aorta and their further differentiation towards ECs (Sato, Watanabe et al. 2008).

The primary function of the Notch signalling during vascular development is to drive the decisions of the precursors between two alternative fates. The Notch signals regulate the specification of arterial or venous identity of ECs, which is achieved by the cross-talk with VEGFA signalling (Lawson, Scheer et al. 2001, Lawson, Vogel et al. 2002) (Figure **1.5A**). The Notch signalling-deficient mice embryos exhibited defectively formed arteries with excessive vein formation (Gale, Dominguez et al. 2004, Krebs, Shutter et al. 2004). Dll1 and Dll4-mediated Notch signalling directly regulate the expression of the specific arterial EC marker EphrinB2 (Iso, Maeno et al. 2006, Sorensen, Adams et al. 2009). VEGFA acts upstream of the Notch signalling pathway during arterial-venous determination of ECs. VEGFA-deficient embryos show similar arteriovenous malformations like Notch-mutant embryos, which can be rescued from ectopic activation of the Notch signalling pathway (Lawson, Vogel et al. 2002). Some studies showed that VEGF exerts its effect on the Notch pathway for arterial specification through ERK pathway to induce the expression of Dll4 and NOTCH1 (Hong, Peterson et al. 2006). Other studies indicated that the cross-talk between VEGF and the Notch pathway is also mediated by transcription factors FOXC1/FOXC2, which can directly activate the promoters of NOTCH1, NOTCH4, Dll4 (Seo, Fujita et al. 2006). In contrary, transcription factor COUP-TFII suppresses the Notch signalling pathway which leads ECs towards venous fate (You, Lin et al. 2005).

The Notch signalling also plays the central role in coordinating angiogenesis process through controlling the EC specification into tip cells or stalk cells, which is also closely interacted with VEGF signalling (Figure **1.5B**). In response to VEGFA stimulation, tip cells express Dll4 which binds to the Notch receptors expressed in

stalk cells. Dll4-activated Notch signalling downregulates the expression of VEGF receptors in the stalk cells, which leads to the inhibition of stalk cells' sprouting tendency towards VEGF stimulus. Therefore, tip cells actively sprout towards the angiogenic signals while the stalk cells maintain the integrity of the vascular bed (Hellstrom, Phng et al. 2007, Suchting, Freitas et al. 2007). Dll4-blockage or Dll4-Notch interaction disruption result in dysregulated vascular formation due to extensive endothelial over-sprouting (Noguera-Troise, Daly et al. 2006). In addition, JAG1 can act as pro-angiogenic regulator by antagonising the Dll4-induced effects and promotes the sprouting and branching of vessels (Benedito, Roca et al. 2009).

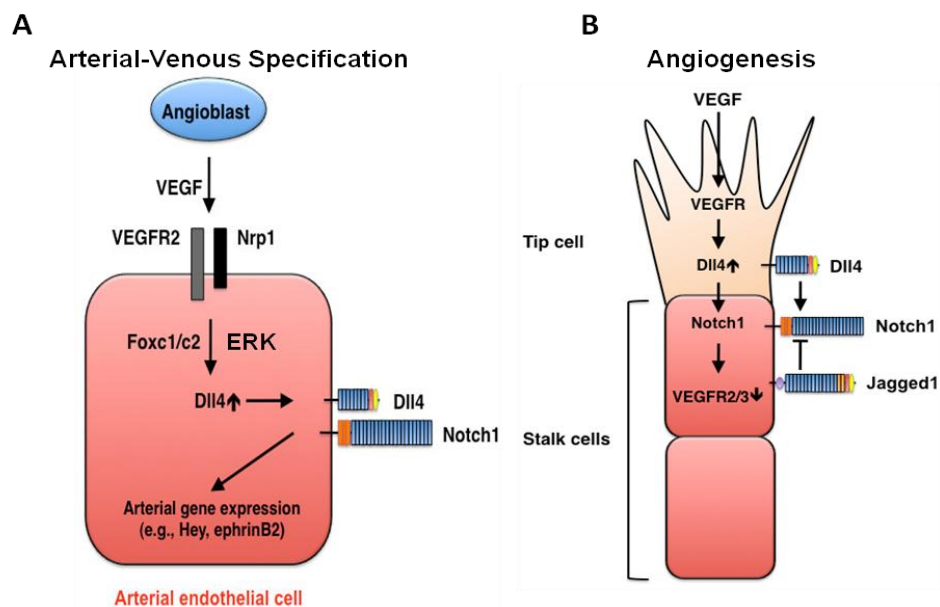


Figure 1.5 The Notch signalling pathway interacts with the VEGF signalling to regulate arterial-venous specification and angiogenesis of EC

(A) The Notch signalling regulates the arterial-venous decision of ECs. In response to VEGF stimulus, the Notch signalling is activated and regulates the expression of arterial genes. **(B)** The crosstalk between the Notch signalling and VEGF signalling controls the angiogenesis process. Tip cells receive VEGF stimulation and start to express high level of Dll4 ligands to inhibit the responses of stalk cells to VEGF. Figure is adapted from (Kume 2009).

The Notch signalling also widely contributes to the SMCs development and physiology. Notch3 is the commonly expressed Notch receptor in SMCs, which is indispensable for the maturation and the acquisition of arterial identity of vascular SMCs. Notch3-deficient mice exhibited fragile arterial structures with abnormally thinner SMCs layer and disorganised elastic lamina compared to the wild-type mice (Domenga, Fardoux et al. 2004). NOTCH3 mutations in human leads to a disease called cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) that is characterised by systemic vascular degeneration due to the extensive SMC death in small arteries (Joutel, Andreux et al. 2000). This suggests that NOTCH3 is important for the maintenance of SMCs phenotypic stability. NOTCH3, together with the downstream HES5, are also implicated in promoting the development of pulmonary arterial hypertension by keeping the SMCs in an undifferentiated state with high proliferation ability (Li, Zhang et al. 2009).

In vitro studies showed that the Notch signalling could either repress or promote smooth muscle gene expression in different context. Ectopic expressing Jag1 or active Notch1 intracellular domain in mouse fibroblasts repressed Myocardin-dependent contractile markers including α -SMA, SMC22 α , Calponin, and SM-MHC (Proweller, Pear et al. 2005). A similar result of extensive suppression of Myocardin-dependent SMC markers was observed by overexpressing NOTCH1 and NOTCH3 intracellular domains in human vascular SMCs (Morrow, Scheller et al. 2005). These suppression effects can be partially explained by the inhibitory roles of Notch downstream target genes of HES/HEY family on the binding of SRF/Myocardin to CArG elements to repress SMC genes (Doi, Iso et al. 2005, Tang, Urs et al. 2008). However, there were reports showing the Notch signalling could induce smooth muscle differentiation and promote the expression of contractile SMC markers (Doi, Iso et al. 2006, Nosedá, Fu et al. 2006). Thus, these contradicting results suggested a dynamic regulatory role of the Notch signalling during SMC development and following injury. The eventual outcome depends on the efficacy and timing of these competing transcriptional events.

In addition, the Notch signalling mediates the cross-talk between ECs and SMCs. The expression of Jag1 on ECs is important for the adjacent SMCs development and maturation. Endothelial-specific deletion of Jag1 caused severe defects in vascular

SMC maturation (High, Lu et al. 2008). Furthermore, the direct cell-cell contact between ECs and SMCs is requisite for the effective conduct of the Notch signalling (Xia, Bhattacharyya et al. 2012). In addition, Jag1 expressed by vascular SMCs can activate Notch1 on ECs to promote proliferation and angiogenesis (Yang and Proweller 2011).

1.2 Vascular Cells Dysfunction leads to a typical vascular pathological condition: Atherosclerosis

1.2.1 Basic pathogenesis of Atherosclerosis

Atherosclerosis is the chronic pathological condition of the arterial wall. It is the major pathological event for many cardiovascular diseases such as coronary artery disease, peripheral artery disease and stroke. The pathogenesis of atherosclerosis is a complex process with the participation of the vascular, metabolic, and immune systems (Loscalzo 2010). Atherosclerosis can be mainly divided into the following stages based on the pathological changes: initial lesion, fatty streak stage, preatheroma stage and atheroma stage (Figure 1.6). Endothelium injury and dysfunction are the initial events induced by genetic and/or environmental risk factors. Then, circulating blood leukocytes adhere to the activated endothelium surface and infiltrate into the tunica intima followed by lipid uptake and foam cells formation. As atherosclerosis deteriorates, SMCs migrate from tunica media into intima, proliferate and produce extracellular matrix macromolecules that form a fibrous cap covering the plaque. The dead SMCs and macrophages debris along with lipid deposition constitute the necrotic core of the plaque. The plaque causes the narrowing of the vascular lumen which leads to local tissue ischaemia. The rupture of the atherosclerotic plaque causes the ultimate complication of atherosclerosis, thrombosis, which obliterates blood flow *in situ* or at the distal ends (Libby, Ridker et al. 2011).

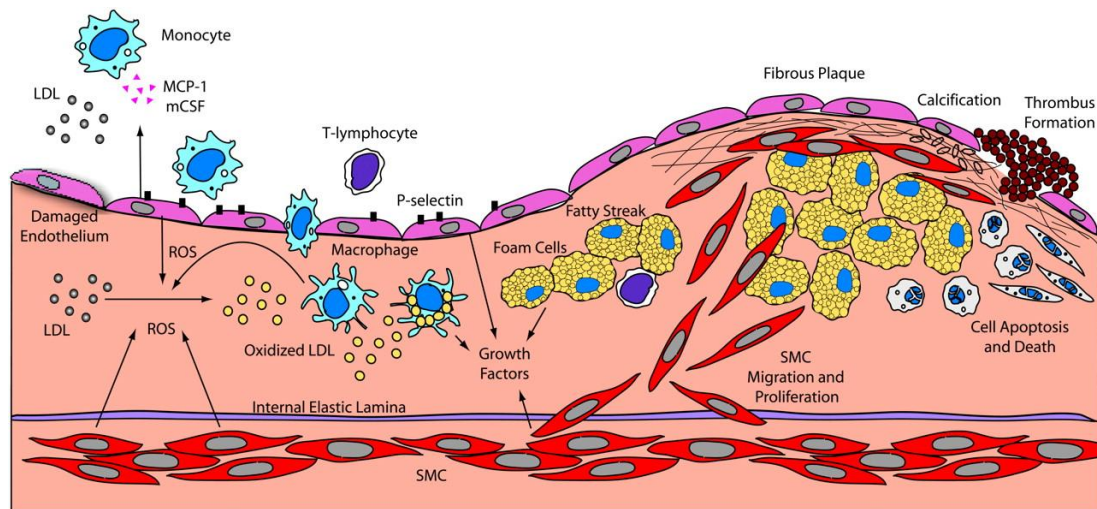


Figure 1.6 A simplified diagram of atherosclerosis pathogenesis

Atherosclerosis is a complex pathological condition featured by endothelial dysfunction, SMC migration and proliferation, lipid deposition, necrotic core and fibrous cap formation. Figure is adapted from (Madamanchi, Vendrov et al. 2005).

1.2.2 Roles of Vascular Cells in Atherosclerosis

Both ECs and SMCs profoundly contribute to the progression of atherosclerosis. The dysfunction of the endothelial monolayer is the key initiation event of atherosclerosis caused by atherogenic stimuli including hypertension, diabetes, dyslipidemia, oxidative stress and others. Endothelial dysfunction characterised by leukocytes recruitment and platelet aggregation, increased permeability, thrombus formation, and impaired endothelium-mediated vasodilation. The expression of surface adhesion molecules are changed within the injured ECs which initiate the recruitment of blood leukocytes and platelet. In parallel, the endothelium permeability and the sub-endothelial extracellular matrix composition are altered to permit the penetration and accumulation of leukocytes and oxidised LDL particles (Landmesser, Hornig et al. 2004). The NO synthesis capacity of ECs is also disturbed during atherosclerosis which impairs the endothelium-dependent vasodilation. Reduced NO synthesis occurs simultaneously with the increased generation of reactive oxygen species (ROS). Increased ROS bioavailability, together with the dysregulated oxidative stress, further damages the endothelial homeostasis (Endemann and Schiffrin 2004). To repair the injured endothelium and

reconstruct normal endothelial physiological function is a major target for anti-atherosclerosis therapy.

SMCs undergo phenotypic modulation during the atherosclerosis lesion formation and progression. In response to the growth factors and cytokines, contractile SMCs within the vessel media layer switch to a synthetic phenotype, characterised with reduced SMC-selective differentiation marker genes and elevated proliferation rate, migratory ability and synthetic activity (Gomez and Owens 2012). Activated SMCs migrate towards the intima where they proliferate and produce extracellular matrix to contribute to the formation of the neointima and the fibrous cap. Up to 70% of the mass of lesions is composed of SMCs and SMC derived cells (Raines and Ross 1993). A recent lineage tracing study showed evidence that SMCs within atherosclerotic lesions could convert to diverse phenotypes expressing markers that are normally expressed by macrophages, mesenchymal stem cells (MSCs) and myofibroblasts (Shankman, Gomez et al. 2015). This study also demonstrated that selective loss of KLF4 profoundly contributes to the generation of SMC-derived macrophage- and MSC-like cells.

1.3 Resident Endothelial Cells Regeneration for Endothelium Repair

After endothelial dysfunction and denudation during atherosclerosis, endogenous resident ECs tend to proliferate and replace the injured endothelium. A number of studies from early years showed that local ECs participate in the repair of small areas of endothelial damage through migration and the repair of larger areas of damage through both proliferation and migration (Hagensen, Vanhoutte et al. 2012). Recently, by transplanting wire-injured carotid artery segments from wild type mice into *Tie2*-GFP mice, Hagensen *et al.* demonstrated the resident ECs from the transplanted graft contribute to the re-endothelialisation of the lesion (Hagensen, Raarup et al. 2012).

Although the proliferation and migration of resident ECs represent a straightforward way for endothelial regeneration, it is a relatively slow and inefficient process (Hirase and Node 2012). In addition, with the effects of the cardiovascular risk factors, the adjacent ECs around injured endothelial area may also be in a dysfunctional state.

The rejuvenation of ECs with normal function represents a significant target of vascular disease therapies. A number of different stem cell sources, that have been considered to contribute to EC regeneration, potentially provide promising methods for regenerative medicine.

1.4 Adult Stem Cells and Endothelial Regeneration

1.4.1 Circulating Vascular Progenitor Cells

Circulating vascular progenitor cells refer to the cells in peripheral blood that can differentiate into ECs and termed as Endothelial Progenitor Cells (EPCs) (Asahara, Murohara et al. 1997). A number of studies demonstrated that EPCs play important roles in vascular healing and remodelling processes.

In general, EPC refers to an immature cell population existing in circulating blood that commits into endothelial lineage and possesses vasculogenic property. There are a motley variety of methods to identify and obtain EPCs and these can be mainly classified into two approaches. For the first approach, mononuclear cells are collected from human peripheral blood or cord blood and then cultured and selected using different *in vitro* culture conditions or colony assays. The second approach is based on the selection of cell subpopulation by surface marker expression. A panel of markers has been used to describe EPCs including CD34, VEGFR2, CD133, and others (Fadini, Losordo et al. 2012, Yoder 2012). Recent evidence shows that EPCs can participate in endothelial repair and compensatory angiogenesis directly as well as through paracrine effects (Yang, Di Santo et al. 2010). Furthermore, the level of circulating EPCs has been used as a biomarker that correlates with the progression of cardiovascular disease (Sen, McDonald et al. 2011). Several early stage clinical trials showed the therapeutic benefit of EPCs for revascularisation in atherosclerotic cardiovascular disease patients (Trounson, Thakar et al. 2011).

Although the concept of EPC has been widely studied and many different methods have been developed to obtain EPCs from human body, the exact characterisation and identification of EPCs are still ambiguous and under debate. Firstly, it is hard to tell whether EPCs grew out of *in vitro* assay actually exist in the circulatory system or they are merely an artificial phenotype generated by certain culture conditions (Fadini, Losordo et al. 2012). Due to the lack of unique or specific markers, it is difficult to distinguish EPCs from subsets of haematopoietic cells, blood platelets or mature ECs (Yang, Di Santo et al. 2010). Another major limitation is that the level of EPC in patient peripheral blood is low. Finally, these so-called EPCs that grew out of circulating blood does not display superior functional properties compared to

mature ECs, questioning their identity as true progenitor or cells that are merely ECs sloughed off from vessel wall (Tura, Skinner et al. 2013). In conclusion, the application value of EPCs in a broad spectrum is still under discussion.

1.4.2 Vascular Wall Resident Progenitor Cells

A variety of resident progenitor cells that can differentiate into endothelial lineage cells have been identified in different sized vessels and all layers of the vessels.

Ingram *et al.* selected endothelial progenitor cells by their proliferative and clonogenic potential among mature ECs in human adult vessel tunica intima (Ingram, Mead et al. 2005). Intima-derived EPCs express similar group of surface markers of ECs including PECAM-1, CD141, CD105, CD146, VE-Cadherin, VWF, and Flk-1. Another study reported a PECAM-1-positive, CD45-negative progenitor population at the inner surface of pre-existing mouse blood vessels (Naito, Kidoya et al. 2012). These EPCs possess colony-forming ability and can give rise to ECs which lead to *de novo* reconstitution of functional blood vessels in ischaemic lesions. EPCs within the intima have the potential to proliferate and may directly contribute to the local migration and repair of damaged endothelium.

A Sca-1-positive, c-Kit-negative/low, Lin28-negative, and CD34-negative/low population of progenitor cells can be isolated from the tunica media of mouse arteries, which can give rise to functional ECs and SMCs *in vitro* (Sainz, Al Haj Zen et al. 2006). In the region between the media and the adventitia of human thoracic aortas and femoral arteries, Pasquinelli *et al.* showed the presence of CD34-positive and c-Kit-positive resident progenitor cells that can develop angiogenic capacity upon culture (Pasquinelli, Tazzari et al. 2007).

The adventitia layer of the vascular wall serves as an important stem cell niche. By using different combination of progenitor markers such as Sca-1, CD34, CD133, VEGFR2 and others, the existences of the multipotent vascular progenitors and EPCs within the adventitia of human and mouse have been demonstrated by many studies (Majesky, Dong et al. 2012). For example, Zengin *et al.* reported that the adventitial layer of human internal thoracic artery contains CD34-positive EPCs that can give rise to capillary-forming ECs *in vitro* and *in vivo* (Zengin, Chalajour et al.

2006). In response to environmental and pathological stimuli, adventitia progenitor cells migrate and differentiate into vascular cells to participate various physiological or disease processes.

The existence of abundant progenitor populations within the vascular wall provides promising prospects for vascular cell regeneration. However, there are no specific markers to define these progenitors while at the same time the origins of these populations are not well understood. In addition, it is difficult to rule out the possibility that the dedifferentiation and transdifferentiation of mature vascular cells are mistaken for novel progenitor population. For therapeutic purpose, how to efficiently and precisely induce the migration of vascular wall progenitors towards specific location for vascular repair is not yet clear.

1.4.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first isolated from bone marrow stroma, and then were reported to be presented in vascular walls, microvessels and perivascular adipose tissues. A panel of markers have been used to define the MSCs: positive for CD105, CD73, CD29, CD90 and CD44, and negative for PECAM-1, CD34, CD45 and CD14 (Uccelli, Moretta et al. 2008). MSCs have multi-lineage differentiation ability including towards ECs. Oswald *et al.* showed that human bone marrow-derived MSCs differentiated towards angiogenic endothelial-like cells by simply culturing the cells in the presence of 2% fetal calf serum and 50 ng/ml VEGF (Oswald, Boxberger et al. 2004). Bone marrow-derived MSCs exhibited the ability to differentiate to both ECs and SMCs and facilitated revascularisation in a canine chronic ischaemic model (Silva, Litovsky et al. 2005).

Clinical advantages of MSCs include the ease of isolation and expansion of the cells and the low immunogenicity (Huang and Li 2008). However, there are some obstacles in the way for the further applications of MSCs especially for vascular regeneration use. MSCs still lack accurate definition due to the absence of one or a group of specific MSC markers. MSCs isolated by different methods show varied differentiation capacities (Gomez-Gavero, Lovell-Badge et al. 2012). In addition, the

true mesoderm lineage differentiation ability of MSCs is still under debate because of the lack of solid lineage tracing experiments (Bianco, Robey et al. 2008).

Adult stem cells are able to give rise to vascular cells under normal and/or pathological conditions (Figure 1.7). However, their therapeutic values are compromised due to the limited population in human body as well as restricted replicative capacity and culture senescence, especially from adult patients. The definition and origin of many kinds of adult stem cells are also far from clear. Thus, it is of significant to explore more cell sources that can be used in vascular regenerative medicine. The discovery of embryonic stem cells offers a thrilling new option.

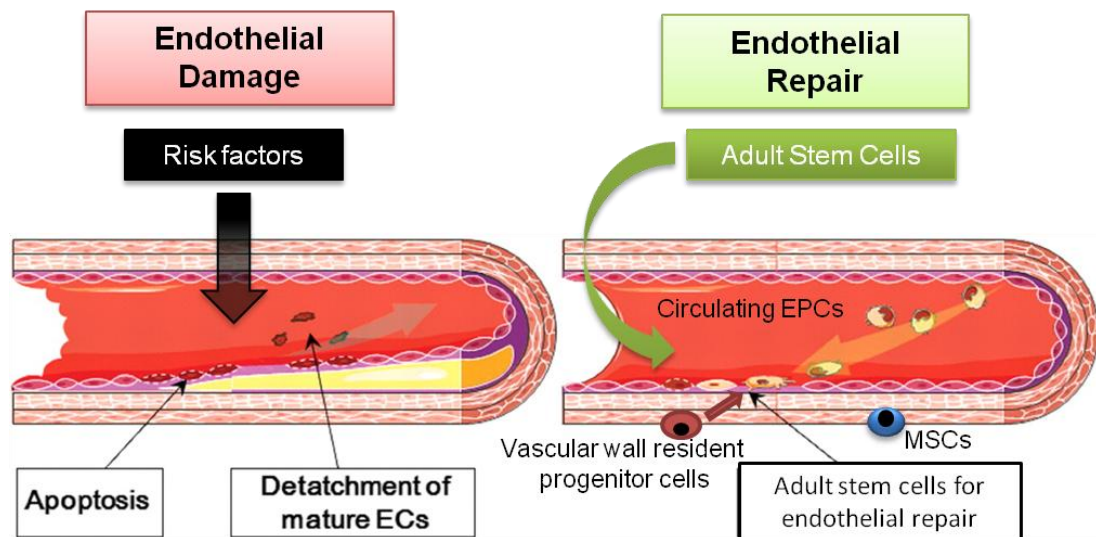


Figure 1.7 Adult stem cells serve as EC reservoirs

In adult body, multiple stem cell sources can contribute to endothelial repair including circulating stem cells, vascular wall resident progenitor cells, mesenchymal stem cells. Figure is adapted from (Fadini and Avogaro 2010) .

1.5 Embryonic Stem Cell (ESC) and Endothelial Regeneration

1.5.1 The Generation of ESC

The successful generation of mouse embryonic stem cells (ESCs) was first reported in 1980s and human ESCs in 1990s, which marked momentous breakthroughs in stem cell research area (Evans and Kaufman 1981, Martin 1981, Thomson, Itskovitz-Eldor et al. 1998). ESCs are isolated from the inner cell mass (ICM) of blastocyst-stage embryos and can be cultured on feeder cells *in vitro*. ESC displays indefinite self-renewal, high proliferative capacity and the ability to differentiate into all cell types of the three germ layers: endoderm, mesoderm and ectoderm, rendering ESC as a promising cell source for regenerative medicine. Both mouse and human ESC are positive for alkaline phosphatase (AP) staining and express pluripotency markers including Oct3/4, Nanog, and Sox2. Nevertheless, there are several distinct characteristics of human ESC from mouse ESC: (1) different signalling networks to regulate the maintenance and the differentiation, especially human ESCs do not require leukaemia inhibitory factor (LIF) to keep the undifferentiated state; (2) expression of specific surface markers like SSEA-3/4, TRA-1-81, TRA-1-60; (3) longer proliferative cycle; (4) different colony shapes (Ginis, Luo et al. 2004, Schnerch, Cerdan et al. 2010).

In culture, ESCs start to differentiate upon removal from feeder cells or LIF. In general, there are three basic approaches for mouse or human ESCs differentiation towards certain lineage: (1) inducing ESCs to aggregate and form 3D structured embryoid bodies (EBs) which further differentiate towards different cell types; (2) culturing ESCs on specific matrix surfaces with defined chemical conditions; (3) co-culturing ESCs with other cell types (Keller 2005).

1.5.2 ESC as a Cell Source for Endothelial Regeneration

Number of studies successfully generated vascular lineage-committed cells from ESCs. The commonly used strategy is a stepwise protocol to differentiate ESCs first towards mesoderm specification and then vascular progenitor cells and finally

mature vascular cells including EC and SMC. CD34, Sca-1, and VEGFR2 are widely used markers to identify the vascular progenitor cells derived from ESCs which can give rise to both EC and SMC that are further capable of producing vessel-like structures (Yamashita, Itoh et al. 2000, Xiao, Zeng et al. 2006, Ferreira, Gerecht et al. 2007, Xiao, Zeng et al. 2007) (Figure 1.8).

Human ESC is considered to be a promising source for functional ECs for further therapeutic applications. By using the EB formation model of human ESCs, endothelial lineage cells can be isolated and propagated at different time points along differentiation. Levenberg *et al.* reported that CD34-positive or PECAM-1-positive cells, sorted from 10 to 13-day old EBs followed by culturing in VEGF supplemented EGM-2 media, could give rise to functional ECs (Levenberg, Golub et al. 2002, Levenberg, Ferreira et al. 2010). Another study segregated PECAM-1-positive, Flk-1-positive, VE-Cadherin-positive, and CD45-negative cell population from day 10 human EB and demonstrated that these precursor cells could commit into functional ECs in endothelial-inducing conditions containing pituitary extracts and VEGF (Wang, Li et al. 2004). Although isolating endothelial lineage cells from EB of different stages is a relatively straightforward method, human EBs are difficult to handle and the efficiency of EC differentiation is generally low (1%-3%).

Another approach is to co-culture human ESCs with feeder cells to induce endothelial lineage generation. Upon culturing on mouse embryonic fibroblasts in differentiation media for 10 days, 5-10% of differentiating human ESCs started to express CD34 which represented a vascular progenitor population. Sorted CD34-positive cells were capable of differentiating into ECs in endothelial growth media (Wang, Au et al. 2007). Other methods including co-culture on OP9, S17, M210 and other mouse fibroblasts feeder cells could also successfully induce CD34-positive vascular progenitor population and further mature functional ECs (Woll, Morris et al. 2008, Hill, Obrtlíkova et al. 2010). However, one major obstacle associated with this kind of approach is the possible contamination of animal products to human cells.

Many groups also established feeder cell-free monolayer culture system to efficiently generate ECs from human ESCs on acellular matrix. After seeding human ESCs on Matrigel and treating with bone morphogenetic protein 4 (BMP4) and inhibitors for MEK/ERK pathway for 3 days, ESCs differentiated into mesoderm-lineage cells

expressing *T* and *WNT3*. Additional 6 days treatments with VEGF and bFGF induced CD34-positive cells arising from the whole population. These CD34-positive progenitor cells could further differentiate into functional ECs when cultured in EGM-2 supplemented with VEGF and bFGF (Park, Jun Koh et al. 2010). Another study showed that culturing human ESCs on fibronectin with defined endothelial differentiation media for 14-21 days could stimulate the generation of tube-forming ECs (Kane, Meloni et al. 2010).

In general, human ESC derived EC (ESC-EC) shares key features with mature EC as the expression of a set of typical EC markers and endothelial physiologically relevant functions such as angiogenesis and vasculogenesis capacity, NO synthesis and release, acetylated-LDL uptake, activation by inflammatory cytokines (Wong, Huang et al. 2012). Furthermore, human ESC-ECs display therapeutic potential in various animal disease models. For example, using the mouse hindlimb ischaemia model, Cho *et al.* showed human ESC-ECs could improve blood perfusion and limb salvage by promoting postnatal neovascularisation (Cho, Moon et al. 2007). Another study demonstrated that human ESC-ECs improved cardiac function and reduced fibrotic scar tissue in a rat model of myocardial infarction (Prado-Lopez, Conesa et al. 2010).

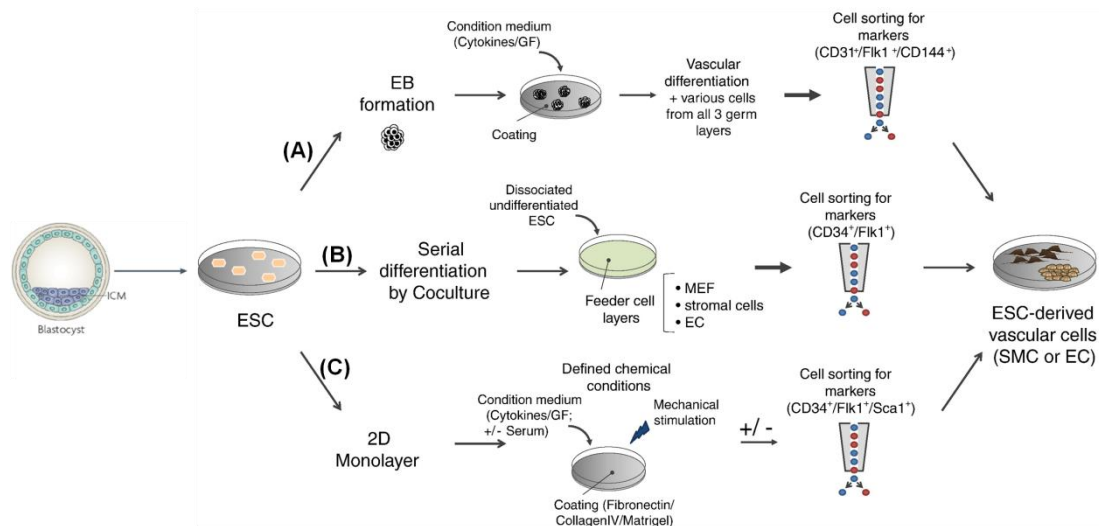


Figure 1.8 Schematic overview of differentiating ESC towards vascular cell lineages

ESC can be differentiated towards vascular lineages through three approaches: **(A)** EB formation; **(B)** co-culture with feeder cells; **(C)** culture with defined chemical conditions. The vascular lineage inductions are usually based on the combined strategy of vascular marker selection and chemical condition stimulation. Figure is adapted from (Hynes 2008, Descamps and Emanuelli 2012).

Although ESC show strong potential for therapeutic purposes, the clinical applications of ESC-derived cells is procrastinated because of several major issues including ethical dilemma, tumourigenesis risk and immunological barriers. Due to these obstacles, ESC-ECs have not been tested in any clinical circumstances with patients. Therefore, ESC is still not an ideal resource for endothelial regeneration therapies.

1.6 Induced Pluripotent Stem Cell (iPS Cell) and Endothelial Regeneration

1.6.1 The Generation of iPS cell

In 2006, Takahashi and Yamanaka first described the process of converting a lineage committed somatic cell back to the pluripotent state by simultaneously overexpressing four transcription factors *Oct4*, *Sox2*, *Klf4* and *c-Myc* using viral vectors (Takahashi and Yamanaka 2006). This Nobel Prize-winning hallmark study successfully reprogrammed mouse fibroblasts to a new type of pluripotent cell that highly resembled mouse ESC in morphology, proliferation, gene expression and DNA methylation patterns. The newly generated cell population was termed “induced pluripotent stem cell” or iPS cell (Figure 1.9).

The following year, the derivation of human iPS cell from human fibroblast was achieved by using similar strategy. Yamanaka’s group generated human iPS cell with the identical set of four transcription factors for the generation of mouse iPS cell (Takahashi, Tanabe et al. 2007). At the same time, Thomson’s group managed to produce human iPS cells using another combination of factors of *OCT4*, *SOX2*, *NANOG* and *LIN28* (Yu, Vodyanik et al. 2007). Human iPS cell exhibit the cellular characteristics that are highly similar to human ESC.

Since then, iPS cells have been successfully generated from different somatic cell types with different combinations of reprogramming factors and various induction methods, which proved the universality of the concept of cell reprogramming (Yamanaka 2012). Till now, a variety of mouse or human cell types have been used to generate iPS cells such as neural progenitor cells, keratinocytes, hepatocytes, B cells (Stadtfield, Brennand et al. 2008, Stadtfield, Nagaya et al. 2008, Zhu, Li et al. 2010). Key transcriptional factors including *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, and *Lin28* were demonstrated to be crucial in inducing iPS cell, although different permutation and combination of these factors can be applied. Regarding gene delivery strategy, retroviral or lentiviral vectors were initially used to deliver genes into somatic cells for iPS cells generation. However, viral transfection might lead to unpredictable genetic dysfunction or insertion mutagenesis (Kaji, Norrby et al. 2009). Therefore, methods to generate iPS cells without viral integration have been

explored including using plasmid, adenovirus, synthesised RNAs, piggyback transposon, and chemicals and small molecules (Yamanaka 2012).

1.6.3 iPS cell-derived EC (iPS-EC) for Vascular Regeneration

iPS cells have the potential to differentiate towards vascular cell lineages including SMC and EC. Due to the similarity shared by iPS cells and ESCs, ECs can be derived from iPS cells by using similar strategies to differentiate ESC to the endothelial lineage: EB formation, coculture with feeder cells or defined chemical conditions. In 2009, two groups first showed that ECs could be generated from human iPS cells with comparable efficiency with ESC-derived ECs. Choi *et al.* cocultured different human iPS cell lines with OP9 feeder cells for 8 days and then selected CD34- and PECAM-1- double positive cell population which could give rise to functional ECs after 7 days under endothelial-promoting culture conditions (Choi, Yu *et al.* 2009). Using a similar approach, Taura *et al.* cocultured human iPS cells with OP9 feeder cells for 10 days and observed the emergence of a VEGFR2-positive population with EC differentiation capacity (Taura, Sone *et al.* 2009). Endothelial lineage-committed cells could also be derived from EB formed by iPS cells (Rufaihah, Huang *et al.* 2011). Most commonly, feeder-free culture systems with the combination of different culture substrates and chemical conditions have been successfully applied to induce ECs from iPS cells (Wong, Huang *et al.* 2012).

iPS-EC displays similar features with mature EC at the genetic and functional level. A major advantage of using iPS cells as EC source is the abundant origins of iPS cells and the potential to generate patient individualised ECs that bypass the immunogenicity and ethical issues. iPS-ECs have been tested in peripheral vascular disease mouse model to show their neoangiogenic capacity that led to the improvement of blood perfusion of ischaemic tissue (Rufaihah, Huang *et al.* 2011).

In spite of the fact that iPS cells start a new era of regeneration medicine, the issue with possible tumour formation hangs dark shadow over their further clinical applications. The fact that many reprogramming factor cocktails contain oncogenes and many gene delivery methods use viral vectors raise the risk of tumour formation

in vivo (Grabel 2012). In addition, iPS cell exhibit more genetic and epigenetic instability compared to ESC due to the artificial reprogramming process (Pera 2011). Therefore, there is still a long way to go before the mature utilisation of iPS cells at the bedside.

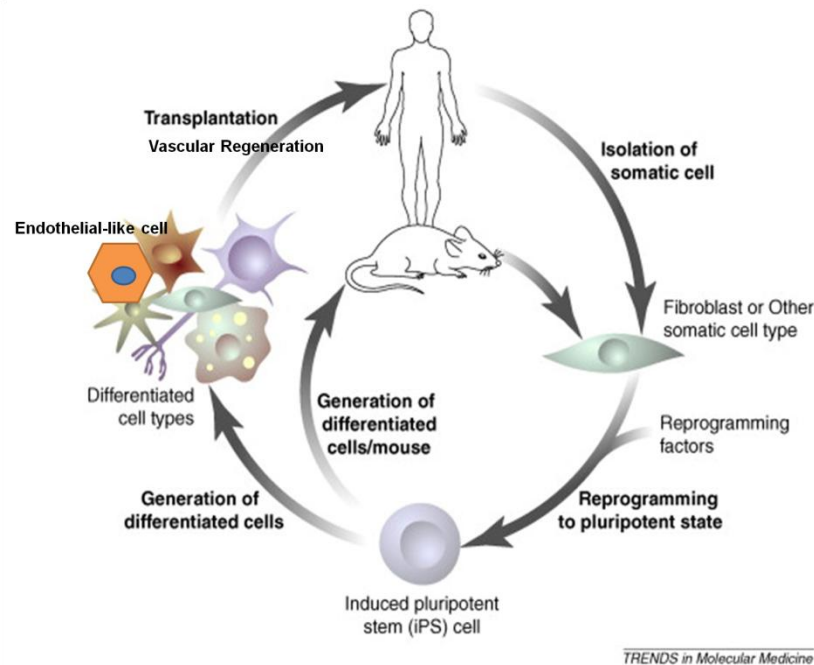


Figure 1.9 The generation and application of iPS cell

iPS cell can be derived from mouse/human somatic cells via reprogramming with transcription factors. Mouse iPS cells can differentiate to distinct cell types of all germ layers as well as the entire mice. Differentiated cells from human iPS cells have the potential to be used for *in vitro* disease modelling and drug screening, transplantation and other applications. Figure is adapted from (Alexander and Bruneau 2010).

1.7 Direct Cell Lineage Reprogramming and Endothelial Regeneration

The first report of direct transdifferentiation from one mature cell type to another was the conversion of fibroblast to skeletal muscle cell via forced ectopic expression of transcriptional factor *MyoD1* (Davis, Weintraub et al. 1987). However, the study of cell lineage conversion progressed slowly until the hallmark study of iPS cell generation and the development of cell reprogramming techniques (Takahashi and Yamanaka 2006). One type of somatic cells can be reverted to iPS cell first, and then differentiate towards another type of somatic cells. Nevertheless, this strategy is time consuming and raises tumour-forming hazards. To avoid issues associated with iPS cell generation, there was a revival in exploiting direct cell fate conversion between two differentiated cell types without passing through the pluripotent state (Figure 1.10).

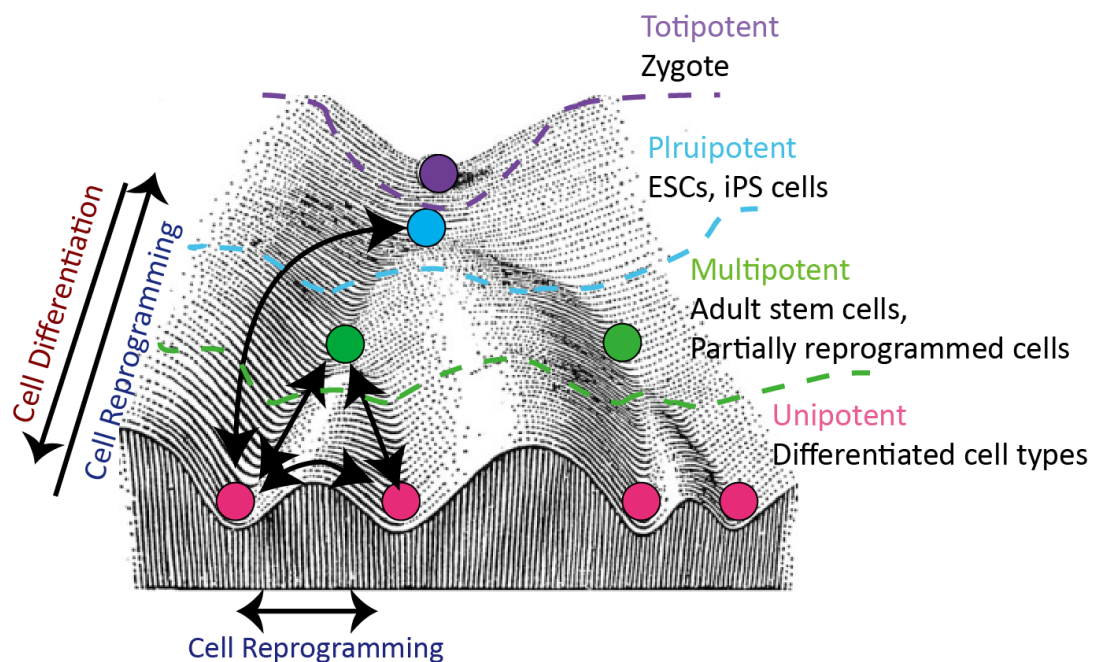


Figure 1.10 The differentiation potential and epigenetic states of cells at different stages of development

The Waddington epigenetic model is used here to illustrate different states of cells and their mutual conversion. Cell differentiation refers to pluripotent/multipotent cells differentiate into lineage-committed cells. In

contrary, cell reprogramming describes the conversion of differentiated cell towards another cell type, either go back to the pluripotent state or to another differentiated cell type. Alteration of cell fates happens together with extensive epigenetic changes of global gene expression. Figure is adapted from (Hochedlinger and Plath 2009).

Presently, based on the use of transcription factors, there are two dominant reprogramming strategies to achieve direct cell-lineage conversion. One is through introducing various combinations of target cell type-specific transcription factors to directly drive cell lineage switch. In 2008, a case of *in vivo* study demonstrated the direct conversion of pancreatic exocrine cell to functional β -cell by injecting adenoviruses encoding three transcription factors Nng3, Pdx1, and Mafa into adult mice pancreas (Zhou, Brown et al. 2008). In 2010, via overexpressing *Gata4*, *Mef2c*, and *Tbx5*, Srivastava's group directly reprogrammed cardiac fibroblast into functional cardiomyocyte *in vitro* (Ieda, Fu et al. 2010). The same group subsequently showed the *in vivo* reprogramming of murine cardiac fibroblast into cardiomyocyte through intra-myocardial injection of the identical set of the three transcription factors (Qian, Huang et al. 2012). In addition, a variety of reports provided evidence of directly reprogramming fibroblast into other cell types including neurons, hepatocytes, etc (Vierbuchen, Ostermeier et al. 2010, Huang, Zhang et al. 2014).

Direct cell lineage reprogramming has also been applied to generate ECs from other somatic cell types by ectopic expression of EC-specific transcription factors. ETS transcription factors are potent regulators for almost all typical endothelial markers (Dejana, Taddei et al. 2007). Ginsberg *et al.* first reported the direct reprogramming of human amniotic fluid-derived cells into ECs by ETS transcription factors *ETV2*, *FLII*, and *ERG1* together with TGF- β suppression (Ginsberg, James et al. 2012). Within this year, there were two important studies published of directly converting fibroblasts into ECs through overexpressing selected endothelial related transcription factors. Han *et al.* converted adult mouse fibroblast into EC using a cocktail of five transcription factors: *Foxo1*, *Er71*, *Klf2*, *Tal1* and *Lmo2* (Han, Chang et al. 2014). Another study showed that solely overexpressing one ETS transcription factor *ETV2*

is sufficient to induce functional ECs from human fibroblasts (Morita, Suzuki et al. 2015).

Another fast and efficient approach to modulate cell fate is based on the use of iPS-generating pluripotency factors such as *Oct4*, *Sox2*, *Klf4*, *Nanog*, etc to erase lineage particular signatures and reactivate repressed epigenetic network as a first step, but with shorter reprogramming time and different culture conditions to avoid the full induction of pluripotency. After this step, cells revert to an intermediate plastic state which permits further manipulations towards the desired cell types. Short-term reactivation of reprogramming genes *Oct4*, *Sox2*, and *Klf4* plus chemically defined media and cardio-inductive growth factor BMP4 converted embryonic and adult fibroblasts to functional cardiomyocytes. During the conversion, the role of reprogramming factors is to erase the original cell identity via epigenetic mechanisms, instead of directly activate cardiomyocyte-specific genes (Efe, Hilcove et al. 2011).

For the lineage conversion towards an endothelial fate, a study showed that by overexpressing *Oct4*, *Sox2*, *Klf4* and *c-Myc* for 8 days, fibroblasts were reverted to an intermediate CD34-positive mesoderm progenitor state marked by which could be further differentiated towards endothelial or smooth muscle lineages under different stimulating conditions (Kurian, Sancho-Martinez et al. 2013). Our lab demonstrated the generation of functional endothelial- and smooth muscle-like cells from human fibroblast through short-term reprogramming with *Oct4*, *Sox2*, *Klf4*, and *c-Myc* which was followed by respective culture conditions (Margariti, Winkler et al. 2012, Karamariti, Margariti et al. 2013).

Beyond the use of transcription factors, recent studies exploited novel approaches including microRNAs (miRNAs), epigenetic regulators, signal pathways modulators and other small molecules to drive cell lineage conversion (Figure 1.11). Conditionally adding these small molecules into the transcription factors cocktail can boost the efficiency of cell fate switching. Furthermore, some combinations of the small molecules alone could drive direct lineage conversion without the ectopic overexpression of transcription factors (Xu, Du et al. 2015).

Different strategies to direct convert somatic cells to ECs are briefly summarised in Table 1.1.

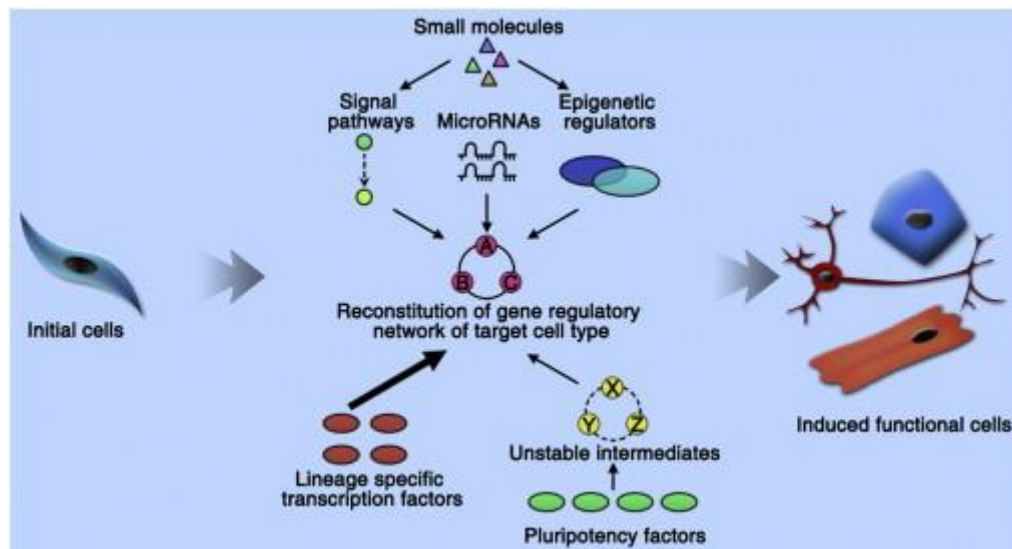


Figure 1.11 Direct cell lineage conversion strategies

Currently, there are two dominant reprogramming strategies to achieve direct cell-lineage conversion. One is through introducing various combinations of transcription factors specific to target cell type to directly drive the cell lineage switch. Another approach is based on the use of iPS cell generating transcription factors with specific culture conditions. The use of microRNAs (miRNAs), epigenetic regulators, signal pathways modulators and other small molecules to carry out direct reprogramming have also been exploited. Figure is adapted from (Xu, Du et al. 2015).

Table 1.1 Studies of the derivation of ECs from other cell types through reprogramming

Cell source/Species	Reprogramming Factors	Reprogramming Conditions	<i>In vitro</i> Characterisation	<i>In vivo</i> application	Reference
Using EC-specific transcription factors for EC reprogramming					
Amniotic fluid-derived cell/Human	<i>FLI1</i> , <i>ERG1</i> (Day 0-Day 21) <i>ETV2</i> (Day 0-Day 14)	Endothelial growth media (medium 199+15% FBS+EC supplement) TGF- β Inhibitor 21 Days	Endothelial marker expression: VE-Cadherin, VEGFR2, PECAM-1; <i>In vitro</i> tubulogenesis	<i>In vivo</i> vessel tube formation assay; <i>In vivo</i> organ-specific vascular education (Liver sinusoidal ECs)	(Ginsberg, James et al. 2012)
Adult skin fibroblast/ Mouse	<i>Foxo1</i> , <i>Er71</i> , <i>Klf2</i> , <i>Tal1</i> , and <i>Lmo2</i>	EBM-2 media+ 10% FBS 12 Days	Endothelial marker expression: VE-Cadherin, PECAM-1, VEGFR2, VWF, Tie2, ICAM2; Ac-LDL uptake; NO production; <i>In vitro</i> angiogenesis	Enhancement of limb perfusion and salvage by implanting into murine model of ischaemic hindlimb	(Han, Chang et al. 2014)

Embryonic lung fibroblast cell line HFL-1/Human	<i>ETV2</i>	Collagen I-coated dish; EGM-2 media with 10 ng/ml VEGF and bFGF; PECAM-1-positive cell sorting on Day 15 and Day 25	Endothelial marker expression: VE-Cadherin, PECAM-1, VEGFR2, VWF, CD34, Tie2; Ac-LDL uptake; <i>In vitro</i> angiogenesis	<i>In vivo</i> angiogenesis assay with Matrigel plugs; Increase of blood flow ratio in murine hindlimb ischaemia model	(Morita, Suzuki et al. 2015)
Using iPS-generating transcription factors for EC reprogramming					
Newborn skin fibroblast cell line: CCD-1079, embryonic lung fibroblast cell line: CCL-186/Human	<i>OCT4, SOX2, KLF4, c-MYC</i> (4 days)	Collagen-IV coated dish; Reprogramming media with 10 ng/ml bFGF for 4 days, then EGM-2 media with 25 ng/ml VEGF for 6 days	Endothelial marker expression: PECAM-1, VE-Cadherin, eNOS, VWF, VEGFR2; Ac-LDL uptake; <i>In vitro</i> angiogenesis	<i>In vivo</i> angiogenesis assay with Matrigel plugs; Increase of blood flow ratio in murine hindlimb ischaemia model; re-endothelialisation in bioengineered vascular graft	(Margariti, Winkler et al. 2012)

Neonatal fibroblast cell line: HFF-1 and BJ, dermal fibroblast cell line: HDF-693/Human	<i>OCT4, SOX2, KLF4, c-MYC, LIN28</i> (\pm miRNAs 302 and 367)(8 days)	Collagen-I coated dish; Plastic induction media for 8 days, then Mesoderm induction media for 8 days, then CD34-positive cell selection followed by EGM-2 media for 8 days	Endothelial marker expression: VE-Cadherin, VWF, VEGFR2, Endoglin; arterial-, venous-, and lymphatic-subtype EC markers; Ac-LDL uptake; <i>In vitro</i> angiogenesis	<i>In vivo</i> angiogenesis assay with Matrigel plugs	(Kurian, Sancho-Martinez et al. 2013)
Neonatal fibroblast cell line: CRL-2097 and BJ/Human	<i>Oct4, Klf4</i>	DMEM/Stemline (1:1) media for 7 days, then Stemline media with BMP4, VEGF, bFGF for 21 days	Endothelial marker expression: VE-Cadherin, PECAM-1, VWF; Ac-LDL uptake; <i>In vitro</i> angiogenesis	<i>In vivo</i> angiogenesis assay with Matrigel plugs	(Li, Huang et al. 2013)
Using other approaches for EC reprogramming					
Neonatal foreskin fibroblasts cell line: BJ/Human	Toll-like receptor 3 (TLR3) agonist Poly I:C (7 days)	Induction media (DMEM+7.5%FBS+7.5% KO serum replacement) for 7 days, then EGM-2 media with BMP4, VEGF, bFGF for 21 days	Endothelial marker expression: VE-Cadherin, PECAM-1, VWF; Ac-LDL uptake; NO production; <i>In vitro</i> angiogenesis	<i>In vivo</i> angiogenesis assay with Matrigel plugs; Increase of blood flow ratio in a murine hindlimb ischaemia model	(Sayed, Wong et al. 2015)

Similar to iPS cell applications, direct cell conversion technique can also be used for patient-specific disease modelling and drug screening. Moreover, an exceptional advantage of direct lineage conversion over iPS cell is the application potential of direct *in vivo* lineage reprogramming for cell replacement therapy, which avoids the unstable long term of *in vitro* cell culture, tumour-forming risks and the technical obstacles for cell transplantation. For example, direct injection of transcription factors cocktail *Gata4*, *Mef2c* and *Tbx5* into the infarcted cardiac area could reprogramme resident non-myocytes into cardiomyocyte-like cells with improved cardiac function (Qian, Huang et al. 2012). The *in vivo* regeneration of functional insulin-producing pancreatic β -cells from other types of pancreatic cells has been reported by several studies for their potential benefits for treating diabetes (Zhou, Brown et al. 2008, Al-Hasani, Pfeifer et al. 2013, Baeyens, Lemper et al. 2014).

1.8 Mechanisms of Cell Reprogramming

1.8.1 Phases of Reprogramming

Since the establishment of reprogramming technique to generate pluripotent iPS cells from lineage-committed somatic cells, researchers have endeavoured to exploit the underlying molecular mechanisms. In general, the complex transcriptional and epigenetic changes of the whole genome occur during the reprogramming to reverse the differentiated cells back to the pluripotent state. Genome-wide analyses of the cell populations at different time points during the reprogramming process revealed three phases for successful iPS cell generation: initiation, maturation, and stabilisation (Samavarchi-Tehrani, Golipour et al. 2010). Each phase characterised by the expression of a distinct group of genes. The initial phase is marked by a mesenchymal-to-epithelial transition. Interestingly, the signature genes associated with the maturation and stabilisation phases are not pluripotency regulators, but rather the genes related to cell cycle, cytoskeletal dynamics and signalling pathways (Golipour, David et al. 2012). Another study also used genome-wide analyses to show that there were two major gene expression changing waves. One occurred between 0-3 days, and the second wave happened between day 9 till the end (Polo, Anderssen et al. 2012). Genes responsible for proliferation and metabolic changes were activated and the genes related to fibroblast identity were suppressed during the first wave. The second wave was characterised by the expression of genes related to stem cell identity establishment and epigenetic remodelling. These cell population-based studies suggested that reprogramming is a multi-step process with transcriptome resetting.

In addition, single-cell studies indicated a stochastic model to describe the reprogramming process: reprogramming factors start a series of probabilistic events that finally lead to only a tiny fraction of cells becoming iPS cells (Hanna, Saha et al. 2009). Buganim *et al.* showed that cell reprogramming process has an early stochastic and a late hierarchic phase by using single-cell gene expression analysis. In the later stage, the activation of *Sox2* starts a sequence of gene expression and eventually results in the acquisition of pluripotency (Buganim, Faddah et al. 2012).

1.8.2 Roles of Reprogramming Factors

In order to elucidate the molecular mechanisms regulating iPS cell generation, it is important to understand the roles of the key reprogramming factors Oct4, Sox2, Klf4, and c-Myc in stimulating pluripotency. All these four factors are vital in the establishment and maintenance of pluripotent state during early embryonic development.

Oct4 is a POU domain-containing transcription factor encoded by *Pou5f1* (Scholer, Ruppert et al. 1990). It is an essential pluripotency regulating gene expressed by early embryo cells. *Oct4*-deficient embryos failed to develop pluripotent cells within the inner cell mass (Nichols, Zevnik et al. 1998). Interestingly, the expression of Oct4 needs to be controlled at a precise level to maintain the pluripotency of ESCs. Either slight suppression or increase of *Oct4* expression leads to distinct differentiation fates of ESCs to mesoderm, endoderm or ectoderm (Niwa, Miyazaki et al. 2000).

Oct4 is arguably the most important factor to induce pluripotency. Oct4 itself can successfully induce pluripotency from somatic cell types that endogenously express canonical reprogramming factors (Kim, Greber et al. 2009, Kim, Sebastiano et al. 2009, Tsai, Bouwman et al. 2011). Single overexpression of Oct4 combined with small chemical molecules could reprogramme fibroblast into iPS cell (Zhu, Li et al. 2010).

SRY (sex-determining region Y)-related high-mobility-group (HMG)-box protein-2, also known as **Sox2**, is a Sox family transcriptional factor involved in the maintenance of ESC pluripotency (Avilion, Nicolis et al. 2003). *Sox2* and *Oct4* act as partners and cooperatively regulate the expression of ESC-specific genes such as *UTF1*, *Fgf4* and *Fbx15* (Scaffidi and Bianchi 2001). *Sox2*-knockout mice die as embryos due to the epiblast lineage failure (Avilion, Nicolis et al. 2003).

The switching on of endogenous *Sox2* expression at the late phase of reprogramming marks the cells that are determined to become iPS cells (Buganim, Faddah et al. 2012). Sox2 governs the establishment of the core pluripotency network in iPS cell.

Krüppel-like factor 4 (Klf4) is expressed in different tissues and widely takes part in regulating cell development, differentiation, proliferation and apoptosis

(McConnell, Ghaleb et al. 2007). *Klf4* is demonstrated to be required for maintaining the pluripotency of ESCs (Jiang, Chan et al. 2008).

c-Myc is a transcription factor involved in various cellular processes including cell growth, cell-cycle regulation, proliferation and differentiation (Schmidt 1999). *c-Myc* participates in the long-term self-renewal of ES cells in culture (Cartwright, McLean et al. 2005).

The clear function of these four transcription factors during cell reprogramming has not been clarified. In general, these four factors act as pioneer factors for remodelling the epigenome. They open up chromatin regions and bind to the promoters of a wide range of genes to guide further epigenetic modification (Buganim, Faddah et al. 2013). Interestingly, in addition to the genes that they usually regulate in ESCs, they also bind to the genes that are not occupied by these factors in ESCs (Soufi, Donahue et al. 2012). This promiscuous binding phenomenon indicated that the roles of the reprogramming factors may be cell type dependent. *Oct4*, *Sox2*, and *Klf4* collectively form a transcriptional network that associates with repressing somatic gene expression and upregulating pluripotent genes during reprogramming. *c-Myc* mainly acts as an transcriptional amplifier at all active promoters to enhance the kinetics and efficiency of reprogramming (Papp and Plath 2011).

The fact that *Oct4*, *Sox2*, *Klf4*, and *c-Myc* are able to open chromatin and induce cell plasticity early in reprogramming support the direct lineage conversion strategy to transiently ectopic express these four factors to dedifferentiate the somatic cell back to a intermediate state followed by further differentiating the cells towards another lineage.

1.8.3 Mesenchymal-to-epithelial transition and Cell Reprogramming

During the phase study for cell reprogramming, a biological process named Mesenchymal-to-Epithelial Transition (MET) has emerged to be a key event for the initial stage of somatic cell reprogramming.

Mesenchymal-state cells and epithelial-state cells are two major animal cell classifications. Epithelial-state cells are closely bound with each other by a variety of cell-cell junctions and align in an apical-basal polarity pattern on the basement membrane. Epithelial-state cells usually express high level of cell junction markers especially E-Cadherin. On the contrary, mesenchymal-state cells represent a loose connected type of cells that lack polarity and can migrate through extracellular matrix (ECM). Typical mesenchymal markers include cytoskeleton components and ECM binding proteins like Fibronectin, Vimentin, N-Cadherin, etc. A phenotypic and morphologic switch from epithelial-state cell to mesenchymal-state cell is called Epithelial-to-Mesenchymal Transition (EMT), which extensively participates in different biological processes including embryonic development and cancer progression (Li, Pei et al. 2014). Some key transcription factors including Snail, Twist, Slug, ZEB have been identified to promote EMT by repressing epithelial-related gene expression (Kalluri and Weinberg 2009). MET is the reverse process of EMT and is characterised by the upregulation of epithelial-related genes especially E-Cadherin and the establishment of cell-cell adhesion along with the downregulation of mesenchymal-related genes. MET has been implicated during embryogenesis and cancer progression (Chaffer, Thompson et al. 2007). Recent studies demonstrated the novel role of MET in somatic reprogramming.

Based on the temporal changes of global gene expression, an important study in 2010 divided the mouse embryonic fibroblast (MEF) to iPS cell reprogramming into three main phases as initiation, maturation and stabilisation. Among which, the initiation stage was characterised by the MET driven by bone morphogenetic proteins (BMPs) signalling (Samavarchi-Tehrani, Golipour et al. 2010). At the same time, another group demonstrated the requisite role of MET at early stage for successful mouse iPS cells generation that was regulated by the interactions with reprogramming transcription factors and TGF- β signalling (Li, Liang et al. 2010).

During the generation of human iPS cell from fibroblast, the participation of MET was confirmed by a study that suggested the reprogramming promoting function of miRNA-302 and miRNA-372 by partly acting on MET (Liao, Bao et al. 2011). Later on, a comprehensive proteomics analysis for the whole course of reprogramming confirmed the existence of MET during early phase at protein level (Hansson, Rafiee et al. 2012). Till now, many studies have proved the occurrence of MET at early stage for successful somatic reprogramming in different cell systems .

Detailed mechanism of the involvement of MET in reprogramming was further investigated (Figure 1.12). Li *et al.* discussed that reprogramming factors *Sox2*, *Klf4*, *Oct4* and *c-Myc* collectively suppress TGF- β signalling which leads to the repression of *Snai1* followed by the activation of E-Cadherin. The same study also showed that *Klf4* can directly activate several epithelial genes including E-Cadherin (Li, Liang et al. 2010). The direct binding of *Klf4* to E-Cadherin promoter region to transcriptionally enhance gene expression is also confirmed in the breast cancer cell line (Yori, Johnson et al. 2010). E-Cadherin-mediated cell-cell contact formation has been shown to be a critical step for the induction of pluripotency from somatic cells (Chen, Yuan et al. 2010). E-Cadherin can even directly replace *Oct4* in reprogramming transcription factors cocktail to achieve successful iPS generation (Redmer, Diecke et al. 2011). It also has been shown that Oct4 and Sox2 could directly activate miRNA-200 which subsequently inhibited ZEB2 and promoted MET for successful reprogramming (Wang, Guo et al. 2013).

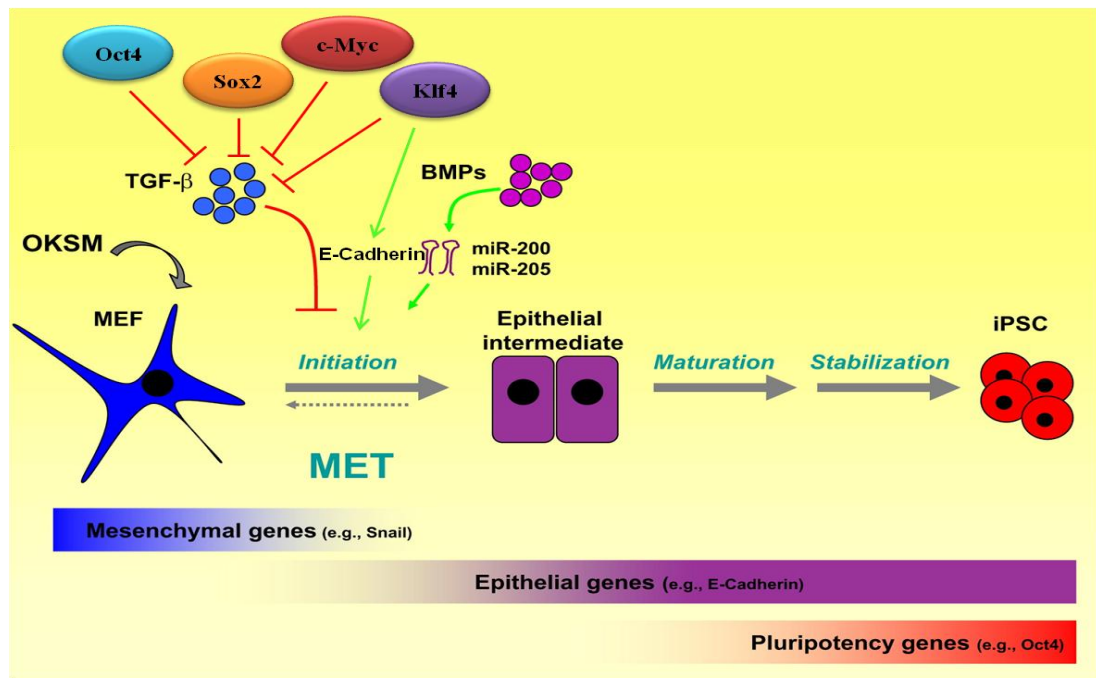


Figure 1.12 MET during the early stage of reprogramming

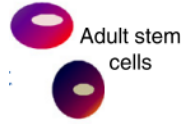
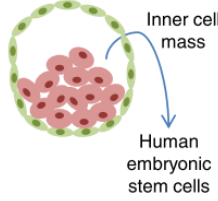
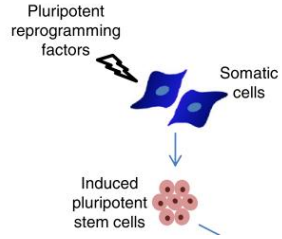
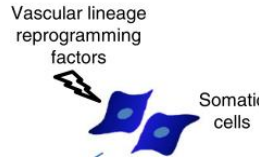
MET is a vital event at the initial stage of reprogramming for successful iPS cell generation. Reprogramming factors coordinate a comprehensive molecular network that leads to the occurrence of MET and the further induction of pluripotent genes. Figure is adapted from (Polo and Hochedlinger 2010).

In addition, recent studies indicated the involvement of MET during the early stage of direct cell lineage conversions, which suggested a more universal role of MET process in modulating cell-identity plasticity. Ectopic introduction of transcription factors *Nr5a1*, *Wt1* and *Dmrt1* into fibroblasts could initiate MET as a first step and finally convert the cells towards embryonic sertoli-like cells (Buganim, Itskovich et al. 2012). Another recent report on directly reprogramming fibroblasts into induced cardiomyocytes demonstrated the involvement of MET (Muraoka, Yamakawa et al. 2014). Promoting MET through suppressing *Snail* by miR-133 could profoundly enhance the protocol efficiency and cell quality of converted cardiomyocytes.

1.9 Comparison of Different Cell Sources for Endothelial Regeneration Purpose

Because of the critical role of EC plays in cardiovascular physiological and pathological conditions, it is of great importance to investigate cells sources that can regenerate functional ECs and further obtain therapeutic value. Early studies attributed endothelial repair exclusively to the proliferation and migration of locally neighbouring ECs after vascular damage (Clopath, Muller et al. 1979). Since the development of the concept of stem cells from late 1990s, other cell sources have been considered to promote endothelial rejuvenation. For now, three major stem cell types are regarded to be promising therapeutic options for endothelial regeneration: EPC, ESC, and iPS cell. In addition, the emergence of direct cell reprogramming provides novel powerful cell sources. The advantages and deficiencies of these cell types and their clinical application value are briefly summarised as follow (Table 1.2):

Table 1.2 Comparison of different stem cell sources for endothelial regeneration

Cell Source	<p><u>Adult Stem Cell</u></p> 	<p><u>ESC</u></p> 	<p><u>iPS Cell</u></p> 	<p><u>Somatic cells</u></p> 
Origin	Circulation, bone marrow or resident tissue	Blastocyst of embryo	Generated by reprogramming of somatic cells, usually fibroblast	Many types of somatic cells: fibroblast, amniotic cell, etc
EC generation	Give rise to EC in response to specific stimulations and endothelial-promoting culture conditions	EB formation and subpopulation selection; culture with feeder cells or specific substrate under chemical defined endothelial-promoting condition	Culture under chemical defined endothelial-promoting conditions; EB formation and subpopulation selection	Reprogrammed by specific transcription factor with endothelial-promoting culture conditions

Main Strengths	<ul style="list-style-type: none"> Autologous Specific endothelial lineage committed Clinical safety 	<ul style="list-style-type: none"> Self-renewal High proliferative capacity 	<ul style="list-style-type: none"> Autologous Self-renewal Large number of cell sources 	<ul style="list-style-type: none"> Autologous Large number of cell sources No tumour formation
Main Weaknesses	<ul style="list-style-type: none"> Ambiguous definition and isolation methods Limited number Limited replicative capacity 	<ul style="list-style-type: none"> Ethical debate Tumourigenesis risk Immunological barriers Unstable cell identity 	<ul style="list-style-type: none"> Tumour formation issue Time consuming Unstable cell identity 	<ul style="list-style-type: none"> Low efficiency Various initial cell types Unstable cell identity
Clinical Application	A number of clinical trials proved the therapeutic benefits for revascularisation and remodelling	No clinical trial data.	No clinical trial data	No clinical trial data

Illustrations are adapted from (Cheung and Sinha 2011).

Many promising alternative cell populations for endothelial regeneration have been explored. However, all these cell types have unsolved problems respectively. Therefore, it is still of great interest to explore other cell types that can regenerate endothelial cells. Cell direct reprogramming techniques provide new possibilities for endothelial rejuvenation.

1.10 Hypothesis, Aims, and Experimental Design of the Study

Rationale: Endothelial denudation and dysfunction underscore many cardiovascular pathological changes and the development of vascular disease. In view of the fact that the spontaneous EC regeneration is a slow and insufficient process, it would be of great significance to explore alternative cell sources that are capable of endothelial regeneration. Stem cells represent promising sources for endothelial regeneration. However, each cell source has its own drawbacks (described in detail in section 1.9). Recent development of cell reprogramming that convert somatic cells directly to other cell types provide new powerful approaches to achieve endothelial regeneration. The original idea to test the feasibility of using SMCs as a new cell source for endothelial regeneration is based on following two points:

- (1) **Significance:** human SMC is an important vascular cell type that underlies the endothelium and composes the majority of vessel wall. In response to endothelial injury, SMCs proliferate and migrate towards tunica intima and accumulate underneath the injured endothelium (Gomez and Owens 2012). If SMC can be converted into functional EC, it may represent a promisingly abundant cell source for *in situ* re-endothelialisation.
- (2) **Feasibility:** developmentally, SMC and EC are both of mesoderm origin. SMCs can be derived from multiple types of progenitor cells during embryonic and postnatal development, among which the existence of vascular progenitors characterised with CD34-positive or VEGFR2-positive that can give rise to both SMC and EC has been reported by many studies (Yamashita, Itoh et al. 2000, Moretti, Caron et al. 2006, Ferreira, Gerecht et al. 2007, Majesky 2007). The fact that SMC shares common progenitor with EC under certain circumstances implies that SMC can be ontogenetically related to EC and share more epigenetic similarity compared to other cell types like fibroblast that has been used to induce ECs (Margariti, Winkler et al. 2012, Kurian, Sancho-Martinez et al. 2013, Morita, Suzuki et al. 2015).

Therefore, the **hypothesis** of this study is to investigate whether functional EC can be derived from human SMC by reprogramming through a plastic vascular progenitor state.

The **aims** of this study are:

- (1) To explore the feasibility of directly converting human SMC towards endothelial lineage through reprogramming.
- (2) Based on the establishment of the protocol, to test the function of the SMC-derived ECs *in vitro* and *in vivo*. Especially their possible application in vascular disease models.
- (3) To explore the possible mechanisms underlying the derivation of ECs from SMCs.

Experimental Design: A combined protocol of reprogramming and differentiation will be applied in this study. In a similar way as previous studies to convert human fibroblasts into functional ECs using short time reprogramming (Margariti, Winkler et al. 2012), SMCs will be reprogrammed by four transcription factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* for a short term to induce a plastic intermediate state. Then, based on the established protocols of differentiating pluripotent or progenitor cell to endothelial lineage, short term reprogrammed-SMCs will be transferred to endothelial-promoting conditions to induce the generation of endothelial-like cells (Figure 1.13). Subsequent studies on endothelial functions and underlying mechanisms will be performed based on the establishment of the protocol.

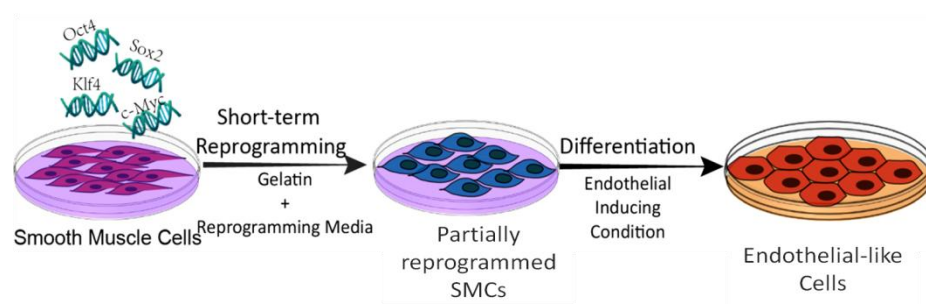


Figure 1.13 Schematic overview of the basic strategy to derive EC from SMC

A combined protocol of reprogramming and differentiation will be applied to covert SMCs towards an endothelial lineage.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Cell culture reagents, chemicals, buffers and kits used in this thesis are presented in Table 2.1.

Table 2.1 Cell culture reagents, chemicals, buffers and kits

	Product	Supplier	Cat. No.
Cell Culture Reagents	SmGM-2 Basal Medium	Lonza	CC-3181
	SmGM-2 SingleQuot Kit	Lonza	CC-4149
	EGM-2 Basal Medium	Lonza	CC-3156
	EGM-2 SingleQuot Kit	Lonza	CC-4176
	KnockOut DMEM/F-12	life technologies	12660-012
	KnockOut Serum Replacement	life technologies	10828-028
	DMEM, high glucose, NEAA, no glutamine	life technologies	10938-025
	Fetal Bovine Serum (FBS)	life technologies	
	Penicillin/Streptomycin	Sigma-Aldrich	P4333
	Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537
	Gelatin Solution 2%	Sigma	G1393
	Mouse Collagen Type IV	BD Biosciences	354233
	0.05% Trypsin-EDTA	life technologies	25300-054
	MEM NEAA 100	Invitrogen	11140
	Human FGF-2	MACS	130-093-837
	Recombinant Human VEGF 165	R&D SYSTEMS	293-VE-010
	β -Mercaptoethanol	Invitrogen	31350-010
	Dimethyl Sulfoxide	Sigma	D5879
	LB Broth	Fisher Scientific	BP1426-2

Chemicals & Buffers	Ampicillin	Sigma-Aldrich	A5354
	FUGENE® HD Transfection Reagent	Promega	E2311
	Polybrene Infection/Transfection Reagent	Merck Millipore	TR-1003-G
	Matrigel Basement Membrane Matrix	BD Biosciences	A6661
	10% SDS Stock Solution	Severn Biotech	CAS 151-21-3
	RIPA lysis buffer	Santa Cruz	sc-24948
	Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
	Precision Plus Protein Ladder	Bio-Rad	161-0374
	Tween 20	Sigma-Aldrich	P2287
	NuPAGE® SDS Running Buffer	life technologies	NP0001
	NuPAGE® Transfer Buffer	life technologies	NP0006
	ECL Western Blotting Detection Reagents	Fisher Scientific	RPN2106
	Paraformaldehyde	Sigma-Aldrich	P6148
	Triton X-100	Sigma-Aldrich	X100
Kits	p24 Antigen ELISA Kit	Perkin Elmer	0801111
	NucleoBond Xtra Midi	Macherey-Nagel	740410
	Basic SMC Nucleofector® Kit	Lonza	VPI-1004
	CD34 MicroBead Kit, human	Miltenyi Biotec	130-046-702
	QIAshredder	Qiagen	79656
	RNeasy Mini Kit	Qiagen	74106
	QuantiTect Reverse Transcriptase Kit	Qiagen	205314
	QuantiFast SYBR Green PCR Kit	Qiagen	74106
	NO cell-based assay kit	Cayman Chemical	10009419

2.2 Methods

2.2.1 Cell Culture

2.2.2.1 Human Umbilical Artery Smooth Muscle Cell (Human UASMC) Culture

Human Umbilical Artery Smooth Muscle Cell (UASMC) line (Lonza, CC-2579) was used as the SMC population in this study and hereinafter referred to SMC. Culture flasks were pre-coated with 0.05% Gelatin (from 2% Gelatin Solution in PBS, both from Sigma Aldrich) for 30 minutes. SMCs were cultured on Gelatin coated flasks in Smooth Muscle Cell Basal Medium (SmBM, Lonza) supplemented with SmBM plus SingleQuots of Growth Supplements (contain EGF; Insulin; bFbF; 5% FBS; Gentamicin/Amphotericin-B. Lonza). Cells were maintained in a humidified incubator with 5% CO₂ at 37 °C.

SMCs were passaged every other day at a ratio of 1:2. Cells were washed in PBS once before Trypsin (0.05% Trypsin-EDTA 1×, GIBCO) was added. The flasks were gently rocked 10-20 times and then the trypsin was discarded. The flasks were kept in the 37° C incubator for 2-3 minutes until cells detached from the flasks. The cells were then resuspended and mixed with medium and transferred to the new Gelatin coated flasks. Cells up to passage 15 were used in this study.

The pictures of live cell morphology were taken with Nikon Eclipse TS100 light microscope and Nikon ELWD 0.3/OD75 camera.

2.2.2.2 Human Embryonic Kidney (HEK) 293T Cell Culture

HEK 293T (ATCC, CRL-11268) cells were cultured in DMEM media with 10% FBS in a humidified incubator with 5% CO₂ at 37 °C. 293T cells were passaged every other day at a ratio of 1:4. Trypsin was used to detach the cells for passaging.

2.2.2 Overexpressing the Four Reprogramming Factors in SMCs using Lentivirus TetO-OSKM

2.2.2.1 Lentiviral TetO -OSKM Construction

A lentiviral construct TetO-FUW-OSKM (TetO-OSKM) (Addgene, Plasmid 20321) containing *Oct4*, *Sox2*, *Klf4*, and *c-Myc* was used to overexpress the four transcription factors in SMCs (Figure 2.1). The four reprogramming factors were separated by 2A sequences in a single FUW lentiviral backbone (Carey, Markoulaki et al. 2009). The empty TetO vector without the encoding sequences of the four factors was used as the negative control vector.

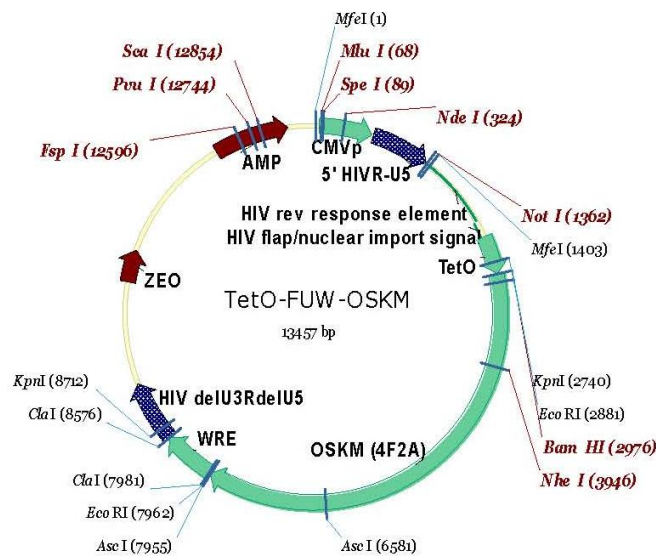


Figure 2.1 Map of the TetO-OSKM vector

Polycistronic lentiviral vector carries the four reprogramming genes *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. Each reprogramming gene is separated by a different 2A sequence (Carey, Markoulaki et al. 2009).

2.2.2.2 Lentivirus TetO-OSKM Production in HEK 293T Cell Line

HEK 293T cells were used to produce lentivirus. 293T cells were co-transfected with the lentiviral vector and the packaging plasmids pCMV-dR8.2 and pCMV-VSV-G (both from Addgene, Plasmid 8455 and Plasmid 8454) using Fugene HD Transfection Reagent (Promega). The supernatant containing the lentiviral particles

was harvested 48 hours after transfection. The supernatant was filtered with 0.45 μ m hydrophilic Polyvinylidene Fluoride (PVDF) membrane filter (Miltenyi Biotec). Then the filtered supernatant containing the lentivirus was aliquoted and stored at -80° C. The viral titers were determined using the p24 antigen ELISA (Perkin Elmer). The Transducing Unit (TU) was calculated based on the conversion from the concentration of p24 to viral titer. For every pg of p24 antigen, there are around 1×10^4 physical particles of lentivirus. Based on ELISA results, there is 100 TU for every pg of p24 antigen (Barde, Salmon et al. 2010).

2.2.2.3 Infection of SMCs with Lentivirus TetO-OSKM

For lentiviral infection, SMCs were seeded on 0.05% Gelatin coated petri-dishes overnight. The following day the cells were incubated with lentivirus TetO-OSKM or control vector (1×10^7 TU/ml) in complete reprogramming media supplemented with 10 μ g/ml of polybrene (Merck Millipore) for 16 hours. Subsequently, media was changed to fresh reprogramming media. Reprogramming media consist of KnockOut DMEM/F12 (life technologies), 20% KnockOut (KO) Serum Replacement (life technologies), 0.1mM β -mercaptoethanol, 0.1 mM MEM Non-Essential Amino Acids (NEAA) (life technologies) and 10 ng/ml basic Fibroblast Growth Factor (bFGF) (Miltenyi Biotec).

2.2.3 Overexpressing the Four Reprogramming Factors in SMCs using pCAG-OSKM Plasmid

2.2.3.1 Plasmid pCAG-OSKM Construction

pCAG-OSKM plasmid (Addgene, Plasmid 20866: pCAG2LMKOSimO) is a polycistronic plasmid originally constructed by Kaji *et al.* in 2009. pCAG-OSKM plasmid contains *Oct4*, *Sox2*, *Klf4* and *c-Myc* gene coding regions linked by 2A peptide sequence driven by CAG enhancer/promoter (Kaji, Norrby et al. 2009) (Figure 2.2). The control empty plasmid was generated from pCAG-OSKM plasmid by removing the four gene-encoding ORF region.

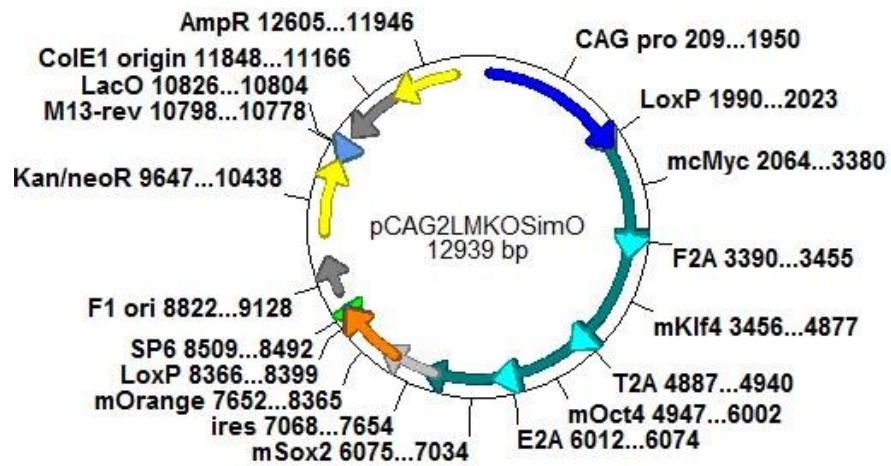


Figure 2.2 Map of the pCAG-OSKM plasmid

Oct4, *Sox2*, *Klf4* and *c-Myc* gene encoding regions are linked by 2A peptide sequences which are driven by a CAG enhancer/promoter in the pCAG backbone. The reprogramming cassette is followed by an ires mOrange fragment (Kaji, Norrby et al. 2009).

2.2.3.2 Restriction Digestions

pCAG-OSKM plasmid was linearised by PvuI restriction enzyme (PvuI, New England BioLabs) at site 12413. Plasmid was incubated with restriction digestion reaction system at 37°C for 3 hours. After linearisation, plasmid was purified with SureClean kit (Bioline).

2.2.3.3 Transfection of SMCs with pCAG-OSKM Plasmid

Transfection of SMCs with pCAG-OSKM or control plasmid was performed using Basic SMC Nucleofector Kit (LONZA) as specified by manufacturer. 2 µg of plasmids was used to transfect every 2×10^6 cells. Cells were first washed with PBS, trypsinised, counted and pelleted. 2×10^6 cells were resuspended with 100 µl Nucleofection solution and then the plasmids were added. The mixture was transferred to a nucleofection cuvette and placed in the LONZA nucleofection machine and the electroporation program for primary SMC line (program A-033) was selected. 1ml warm medium was added into the cuvette soon after the electroporation and mixed gently. The cell solution was transferred to a 0.05% Gelatin coated T25 flask in a drop-wise manner.

2.2.4 Cell Reprogramming

SMCs transfected with the four reprogramming factor-expressing vector or control empty vector were maintained in reprogramming media consisted of Knockout DMEM/F12 (life technologies), 20% Knockout Serum Replacement (life technologies), 0.1 mM β -mercaptoethanol (life technologies), 0.1 mM MEM NEAA (life technologies) and 10 ng/ml bFGF (Miltenyi Biotec). Media was changed every day.

2.2.5 Cell Differentiation towards an Endothelial Lineage

Culture flasks were pre-coated with 5 μ g/ml Collagen IV (BD mouse collagen IV) in PBS for 2 hours at room temperature. After 4 days of reprogramming, partially reprogrammed SMCs (PR-SMCs) were transferred to Collagen IV- coated flasks and maintained in EGM-2 Basal Media supplemented with EGM-2 SingleQuot Kit (contain EGF, IGF-1, VEGF, Ascorbic Acid, Hydrocortisone, bFGF, 5% FBS, Gentamicin/Amphotericin-B. Lonza). Additional 25 ng/ml VEGF (Recombinant Human VEGF₁₆₅, R&D was supplemented to the culture media. PR-SMCs were cultured under these conditions for 6 days to promote endothelial differentiation. Media was changed every other day. SMC-derived ECs (SMC-ECs) were generated after 6 days of endothelial differentiation.

2.2.6 Selection and Culture of CD34-positive Cells from PR-SMCs

CD34 MicroBead Kit (Miltenyi Biotec) was used to select CD34-positive cells from partially reprogrammed SMCs (PR-SMCs) after 4 day reprogramming. PR-SMCs were washed with PBS, trypsinised, counted and pelleted. The cell pellet was resuspended in 300 μ l of MACS Buffer (PBS contains 0.5% bovine serum albumin (BSA) and 2 mM EDTA) to which 100 μ l of FcR Blocking Reagent and 100 μ l CD34 MicroBeads were added. Cell suspension was mixed well and incubated for 30 minutes at 4 °C. After incubation, cells were washed in 6ml of MACS Buffer, pelleted and resuspended in 500 μ l of buffer. Cell suspension was applied to the MS

MACS separation column. Cells that labelled with CD34 Microbeads bind to the column and cells that passed through the column were discarded. The column was then washed three times with 500 µl MACS Buffer each time and then removed from the separator and placed in a collection tube. 1ml of MACS Buffer was applied into the column. The labelled cells were flushed out by firmly pushing the plunger into the column. Isolated CD34-positive cells were grown in T25 flask coated with 5 ng/ml of Collagen IV and cultured in EGM-2 media supplemented with 25ng/ml VEGF (R&D Systems).

2.2.7 Nucleic Acid Analyses

2.2.7.1 Total RNA Extraction

Cells were harvested from the tissue culture flasks by scrapping in ice-cold PBS. Cells were pelleted by centrifuging at 1000 rpm for 5 minutes. The supernatants were discarded. RNA extraction was performed with RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Cells were harvested and disrupted with 350 µl RLT Buffer. Lysate was pipetted into a QIAshredder spin column and centrifuged for 2 minutes to achieve homogenisation. 1 volume of 70% ethanol was added to the homogenised lysate and mixed well to create the optimised condition for the RNA to selectively bind to the RNeasy membrane. The samples were then applied to RNeasy spin column and centrifuged for 30s. Total RNA bound to the membrane and the contaminants were washed away with RW1 and RPE washing buffers. At last, the RNA was eluted with RNase-free water. The concentration of the RNA was measured with the Nanodrop® Spectrophotometer (Thermo Scientific) and the purity of the RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. RNA with an A_{260}/A_{280} ratio value around 2.0 was used in the downstream procedures.

2.2.7.2 Reverse Transcription (RT) Reaction

QuantiTect® Reverse Transcription System (Qiagen) was used as specified by the manufacturer to synthesize cDNA from total RNA. 1 µg of RNA sample was first

mixed with 2 μ l of gDNA Wipeout Buffer and the total volume was adjusted to 14 μ l with RNase-free water. The mixture was incubated at 42 °C for 2 minutes to remove the genomic DNA contaminations. Then, 1 μ l of Reverse Transcriptase, 1 μ l of RT Primer Mix and 4 μ l of RT buffer were added to the reaction system. The mixture was incubated for 15 minutes at 42 °C and then 3 minutes at 95 °C to inactivate the synthesis reaction. With the assumption that all the RNA was converted to cDNA, the final concentration of the cDNA was diluted to 10 ng/ μ l with 80 μ l of RNase-free water.

2.2.7.3 Quantitative Real-Time Polymerase Chain Reaction (Real-Time PCR)

Real-time PCR was performed to detect and quantify gene expression. The sample was prepared with 2 μ g cDNA, 0.75 μ l forward primer (from 2 μ M stock), 0.75 μ l reverse primer (from 2 μ M stock), 10 μ l SYBR Green PCR master mix (Qiagen) and 6.5 μ l RNase-free water. Sequences of the primers used were listed in Table 2.2. ABI Prism 7000 Sequence Detection System instrument (Applied Biosystems) was used to carry out the PCR reaction. cDNA samples in duplicates were denatured at 95° C for 2 minutes, then amplified by 40 cycles at 95° C for 15 seconds and 60° C for 1 minute and finally extended at 72° C for 1 minute. Threshold cycle numbers (Ct) were measured in the exponential phase for all samples. Fold change was calculated as the relative fold difference of Ct value of target gene against GAPDH housekeeping gene.

Table 2.2 Sequences of primers for real-time PCR

Name	Sequence (5'→3')
OCT4 Forward	ATGCATTCAAAGTGGGTGCCTGC
OCT4 Reverse	AACTTCACCTTCCCTCCAACCAGT
SOX2 Forward	CACATGAAGGAGCACCCGGATTAT
SOX2 Reverse	GTTCATGTGCGCGTAACTGTCCAT
KLF4 Forward	TGACCAGGCACTACCGTAAACACA
KLF4 Reverse	TCTTCATGTGTAAGGCGAGGTGGT

c-MYC Forward	ACAGCTACGGAACTCTTGTGCGTA
c-MYC Reverse	GCCCAAAGTCCAATTTGAGGCAGT
α -SMA Forward	TGACAATGGCTCTGGGCTCTGTAA
α -SMA Reverse	TTCGTCACCCACGTAGCTGTCTTT
SM22 α Forward	TTGAAGGCAAAGACATGGCAGCAG
SM22 α Reverse	TCCACGGTAGTGCCCATCATCTT
Calponin Forward	TTGAGGCCAACGACCTGTTTGAGA
Calponin Reverse	TCGAATTTCCGCTCCTGCTTCTCT
SM-MHC Forward	AGAAGCCAGGGAGAAGGAAACCAA
SM-MHC Reverse	TGGAGCTGACCAGGTCTTCCATT
CD34 Forward	CACTGAGCAAGATGTTGCAAGCCA
CD34 Reverse	TCAGGAAATAGCCAGTGATGCCCA
VEGFR2 Forward	ATCCAGTGGGCTGATGACCAAGAA
VEGFR2 Reverse	ACCAGAGATTCCATGCCACTTCCA
PECAM-1 Forward	AGCCCGAACTGGAATCTTCCTTCA
PECAM-1 Reverse	TCCTTCTGGATGGTGAAGTTGGCT
VE-Cadherin Forward	GCCAGGTATGAGATCGTGGT
VE-Cadherin Reverse	CAACAAACAGAGAGCCCACA
eNOS Forward	ACCCTCACCGCTACAACATC
eNOS Reverse	GCTCATTCTCCAGGTGCTTC
VWF Forward	CTGAAGGGCTCGAGTGTACC
VWF Reverse	CACATGGTCTGTGCAGTTCC
Claudin5 Forward	CTGCTGGTTCGCCAACATT
Claudin5 Reverse	TGCGACACGGGCACAG
E-Cadherin Forward	GTCACTGACACCAACGATAATCCT
E-Cadherin Reverse	CAGTGTGGTGATTACGACGTTA
SNAI1 Forward	TCGGAAGCCTAACTACAGCGA
SNAI1 Reverse	AGATGAGCATTGGCAGCGAG
Fibronectin Forward	AAACTTGCATCTGGAGGCAAACCC
Fibronectin Reverse	AGCTCTGATCAGCATGGACCACTT
Mucin1 Forward	AGCTGCCCCGTAGTTCTTTTCG
Mucin1 Reverse	CGCTGGCCATTGTCTATCTCA

Claudin1 Forward	GCGCGATATTTCTTCTTGCAGG
Claudin1 Reverse	TTCGTACCTGGCATTGACTGG
JAG1 Forward	GTCCATGCAGAACGTGAACG
JAG1 Reverse	GCGGGACTGATACTCCTTGA
HES5 Forward	GTCAGCTACCTGAAGCACAGCAAA
HES5 Reverse	TGGAAGTGGTACAGCAGCTTCATC
DLL4 Forward	AGGCCTGTTTTGTGACCAAG
DLL4 Reverse	CTCCAGCTCACAGTCCACAC
NOTCH1 Forward	GTCAACGCCGTAGATGACC
NOTCH1 Reverse	TTGTTAGCCCGTTCTTCAG
NOTCH4 Forward	AGCTGCCCTTGATCTTCCAG
NOTCH4 Reverse	TAATACGACTCACTATAGGG
HEY1 Forward	ATTTTGGCCAGAAAAAGACG
HEY1 Reverse	CGCGTCAAAGTAACCTTTCC
HEY2 Forward	AAGATGCTTCAGGCAACAGG
HEY2 Reverse	ACAAGGGGTTGACTCTGAGG
GAPDH Forward	CATGTTTCGTCATGGGTGTGAACCA
GAPDH Reverse	ATGGCATGGACTGTGGTCATGAGT

2.2.7.4 RNA-Sequencing Analysis

The following groups were analysed: SMCs, HUVECs, PR-SMCs, SMC-ECs and SMC-CD34-ECs. Total RNA was extracted using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA quantity and quality were checked with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and electrophoresis on a denaturing agarose gel. cDNA libraries were prepared using Illumina TruSeq RNA-Seq sample preparation kit (Illumina, San Diego, CA). The libraries were analysed using Illumina HiSeq2500. Results were generated as fastq files and sequence reads were trimmed to remove low quality bases at ends. Then sequence reads were mapped to the reference of Homo sapiens genome using CLC Genomics Server program. Hit counts and reads per kilobase per million (RPKM) values were calculated for genes, which is first to align the raw read counts to exons

of mRNA transcripts in RefSeq and then normalised these values to total uniquely aligning reads and transcript length.

Gene expression analysis was based on the normalised RPKM values of genes, including global gene expression analysis and expression comparisons for individual genes of interest. Differential expression was defined as a minimum 2 folds change. Global gene expression analysis was performed with Cluster 3.0 with average linkage. Genes with the RPKM values below 0.5 were removed from the analysis. The results were visualised with Java TreeView in the form of red-green heat map. Colour red represents the high gene expression level while colour green represents the low gene expression level. Gene Ontology (GO) analysis was performed on the upregulated genes in PR-SMCs or SMC-CD34-ECs compared to SMCs. DAVID bioinformatics resource 6.7 functional annotation tool suite was used to carry out the GO analysis. P-value is calculated by the software and represents the significance of the particular GO term associated with the group of genes. The closer the p-value is to zero, the more significant the GO term is annotated to the genes.

2.2.8 Protein Analyses

2.2.8.1 Total Protein Extraction

Cells were harvested from the tissue culture flasks by scrapping in ice-cold PBS. Cells were pelleted by centrifuging at 1000 rpm for 5 minutes. The supernatants were discarded. Cell pellet was lysed in RIPA Lysis Buffer (1×, Santa Cruz). The lysate was then sonicated twice with Branson Snifier 150 (Emerson Industrial Automation) at lowest setting for 6 seconds and then incubated on ice for 45 minutes. Cell debris was removed by centrifuging at 13,200 rpm for 10 minutes at 4°C. The supernatant which contained the whole cell lysate protein was collected.

2.2.8.2 Quantification of Protein by Bradford Method

To test the concentration of the extracted protein, 2 µl protein lysate from total protein extraction was combined with 998 µl Bio-Rad Protein Assay Dye Reagent (1:5 diluted in ddH₂O) in a 1.5 ml Eppendorf tube and incubated for at least 5

minutes at room temperature. Absorbance at 595 nm was measured with the Bio-Rad SmartSpec™ 3000 spectrophotometer and protein concentration was calculated based on the BSA standard curve.

2.2.8.3 Western Immunoblotting

The extracted protein was mixed with 1/4 volume of 5×SDS Buffer and incubated for 5 minutes at 96° C. Protein samples and Precision Plus Protein Ladder (Bio-Rad) were fractionated by size on NuPAGE® 4%-10% Bis-Tris pre-cast gel (life technologies) by electrophoresis at 160V constant voltage for approximate 1 hour in the running buffer. Nitrocellulose Membrane (Protran, Whatman) was placed against the gel containing the resolved proteins and sandwiched in a transfer cassette (XCell II Blot Module, life technologies) between two layers of filter paper and two layers of foam sponge. The resolved proteins were transferred from the gel to the membrane for 2 hours at 30 V in the transfer buffer (life technologies).

The membrane was rinsed in PBS and blocked in 5% Milk with 0.002% NaN₃ for at least 1 hour at room temperature. The membrane was then incubated with the appropriate primary antibody listed in Table 2.3 overnight at 4° C. After primary antibody incubation, the membrane was washed three times, 15 minutes each in PBS-Tween Solution (0.01% Tween 20 in PBS). The membrane was then incubated with the appropriate secondary antibody for 1 hour at room temperature. Finally, the membrane was washed three times, 10 minutes each in PBS-Tween Solution.

Protein expression was visualised by incubating the membrane with Enhanced Chemiluminescent (ECL) Western Blot Substrate (Amersham ECL Plus Western Blotting Detection Reagents, GE) for 2 minutes and then the chemiluminescent signal was detected by exposure of the membrane to the film (Amersham Hyperfilm ECL, GE) with the Compact X4 X-Ray film processor (Xograph Imaging system).

Table 2.3 Antibodies used in Western Blotting analysis

Primary Antibody	Host	Dilution	Supplier	Cat. No.
------------------	------	----------	----------	----------

α -SMA	Mouse	1:1000	Sigma Aldrich	A5228
SM22 α	Rabbit	1:1000	Abcam	ab14016
Calponin	Rabbit	1:1000	Abcam	EP798Y
SOX2	Rabbit	1:500	Abcam	ab59776
OCT4	Mouse	1:500	Santa Cruz	sc5279
KLF4	Rabbit	1:500	Santa Cruz	sc20691
VE-Cadherin	Rabbit	1:500	Abcam	ab33168
PECAM-1	Rabbit	1:500	ABBIOTEC	252253
eNOS	Rabbit	1:200	BD Biosciences	610297
Claudin 5 (CLDN5)	Mouse	1:500	life technologies	352588
E-Cadherin	Rabbit	1:500	Cell Signaling	3195P
SNAIL	Rabbit	1:1000	Cell Signaling	3879P
N-Cadherin	Rabbit	1:300	Abcam	ab12221
HES5	Rabbit	1:400	Millipore	Ab5708
Jagged 1 (JAG1)	Rabbit	1:300	Santa Cruz	sc8303
GAPDH	Mouse	1:2000	Abcam	ab8245
Secondary Antibody	Host	Dilution	Supplier	Cat. No.
Anti-Mouse Immunoglobulins	Rabbit	1:3000	Dako	P0260
Anti-Rabbit Immunoglobulins	Swine	1:3000	Dako	P0217

2.2.8.4 Immunofluorescence Staining

Cells were seeded on slide and cultured in corresponding medium until achieved 70% confluency. After washing with PBS, cells were fixed with 4% Paraformaldehyde (PFA) made in PBS for 15 minutes and then permeabilised with 0.1% Triton X-100 made in PBS for another 15 minutes. Frozen section samples were fixed in cold acetone for 10 minutes and then washed with PBS for three times. Samples were blocked by 5% serum from the host species of secondary antibodies in PBS for 30 minutes, followed by the incubation with primary antibodies listed in Table 2.4

diluted in 5% serum overnight at 4° C. After washing three times with PBS, samples were incubated with secondary antibodies for 45 minutes at 37° C. Next, samples were washed with PBS three times and then 4'-6-diamidino-2-phenylindole (DAPI) was applied to stain the nuclei for 2 minutes. After further washing with PBS three times, the coverslips were added to the slide with a drop of Fluorescent Mounting Media (Dako). Images were taken by Olympus IX81 microscope equipped with TRITC, FITC and DAPI filters.

Table 2.4 Antibodies used in Immunofluorescence Staining

Primary Antibody	Host	Dilution	Supplier	Cat. No.
SM22 α	Rabbit	1:100	Abcam	ab14016
Calponin	Rabbit	1:100	Abcam	EP798Y
CD34	Mouse	1:50	Santa Cruz	sc7324
eNOS	Rabbit	1:50	BD Biosciences	610297
PECAM-1	Goat	1:50	Santa Cruz	sc1506
VE-Cadherin	Goat	1:50	Santa Cruz	sc6458
VEGFR2	Mouse	1:100	Abcam	ab9530
Human-specific PECAM-1	Rabbit	1:100	Abcam	ab32457
E-Cadherin	Rabbit	1:50	Santa Cruz	sc7870
HES5	Rabbit	1:50	Millipore	Ab5708
α -SMA-Cy3 (conjugated)	Mouse	1:100	Sigma Aldrich	C6198
Secondary Antibody	Host	Dilution	Supplier	Cat. No.
Anti-Mouse Alexa Fluor 488	Donkey	1:500	life technologies	A21202
Anti-Rabbit Alexa Fluor 488	Donkey	1:500	life technologies	A21207
Anti-Goat Alexa Fluor 488	Donkey	1:500	life technologies	A11055
Anti-Mouse Alexa Fluor 594	Goat	1:500	life technologies	A11005
Anti-Rabbit Alexa Fluor 594	Donkey	1:500	life technologies	A21207

2.2.8.5 Flow Cytometry Analysis

Flow cytometry analysis was performed to analyse the percentage of surface marker expression in different cell samples. Cells were harvested and fixed with 4% PFA for 15 minutes and then blocked with 5% serum from the host species of secondary antibodies in PBS for 20 minutes on ice. The specific fluorochrome-conjugated primary antibodies as listed in Table 2.5 were applied to stain the cell surface and isotype-matched IgG antibodies were used as control. After incubation for 1 hour in dark on ice, cells were washed with PBS three times and centrifuged. Cell pellet was resuspended in 1% PFA and keep at 4° C till analysis.

Fluorescence activated cell sorting flow cytometer (Becton Dickinson Immunocytometry system) was used to perform the analysis. Data was collected and analysed with BD CellQuest Pro software. Histograms were used to shown the increase of the percentage of EC surface markers in the differentiated cells compared to the control cells after adjustment for background fluorescence with control isotype-matched antibody-stained cells. The gate M1 region was set with <1% of the peak of the control cells inside the gate's left edge.

Table 2.5 Antibodies for Flow Cytometry Analysis

Conjugated Antibody	Host	Supplier	Cat. No.
Anti-human VEGFR2-FITC	Mouse IgG	R&D	FAB357F
Anti-human CD34-PE	Mouse IgG	MACS	130-081-002
Anti-human PECAM-1-PE	Mouse IgG	Abcam	ab30349
Anti-VE-Cadherin -FITC	Rabbit IgG	Abcam	ab33321
Mouse IgG-PE, Isotype Control	Mouse IgG	MACS	130-091-835
Mouse IgG-FITC, Isotype Control	Mouse IgG	R&D	IC002F
Rabbit IgG-FITC, Isotype Control	Rabbit IgG	Abcam	ab37406

2.2.9 Cell Function Analyses

2.2.9.1 Nitric Oxide (NO) Fluorometric Assay

The NO cell-based assay kit (Cayman Chemical) was used to measure cellular NO level. Fluorescein amine methyl ester (FA-OMe) can form fluorescent deamination product (da-FA-OMe) when senses free NO in living cells. SMC-ECs or empty vector transfected control cells were incubated with FA-OMe staining solution for 24 hours. Cells were then washed with assay buffer and stained with Hoechst Dye for nuclei. The fluorescent signals were then detected by fluorescent microscope.

2.2.9.2 Acetylated-LDL Uptake Assay

70% confluent converted ECs on slide were incubated with 10 µg/ml Alexa Fluor 594 conjugated Ac-LDL (life technologies) for 4 hours in culture media at 37 °C. Cells were then washed with PBS and stained with DAPI. Samples were immediately visualised under fluorescent microscope.

2.2.9.3 *In vitro* Matrigel Tube Formation Assay

100 µl Matrigel (BD Biosciences) was added to each chamber of an 8-chamber slide and allowed to solidify for 1 hour at 37° C. 1×10^5 SMC-CD34-ECs or control SMCs were suspended in 300 µl EGM-2 media supplemented with 25 ng/ml VEGF and added into each Matrigel-coated chamber. Chamber slide was maintained in a humidified incubator with 5% CO₂ at 37° C. Tube formations were assessed and images were taken at the time point of 6 hours with light microscope. Total tube length was quantified with ImageJ processing program with tool Angiogenesis Analyzer. The immunofluorescence staining was then performed similarly to the cell staining described above but with prolonged incubation times each step.

2.2.9.4 *In vivo* Matrigel Plug Assay

5×10^5 SMC-CD34-ECs or control SMCs were mixed well with 250 µl Matrigel and 50 µl EGM-2 media supplemented with 25 ng/ml VEGF. The cell mixture was

injected subcutaneously into the NOD.CB17-*Prkdc*^{scid}/NcrCrl mice where it rapidly solidified. Six injections were conducted for each group. The plugs were harvested 14 days later. Samples were fixed in liquid nitrogen, cryosections were prepared and immunofluorescence staining with PECAM-1 antibody was then performed to detect the capillary structures.

2.2.10 Mouse Hindlimb Ischaemia Model

6-8 weeks old mice were used to create the hindlimb ischaemia model. Mouse was placed at a supine position after anaesthetisation and the medial side of the right hind limb was shaved and disinfected. An approximately 1cm long skin incision was made from the knee towards the medial thigh. Next, the femoral artery and vein were exposed and dissected from the surrounding connective tissue. Then the femoral artery was separated from the vein and ligated twice at the distal and proximal ends. Then the artery was dissected between the two ligations. The paleness level of the foot was checked to confirm the successful induction of limb ischaemia. Then the incision is closed by sutures.

SMC-CD34-ECs or empty vector transfected SMCs (control) were injected intramuscularly into the adductors of ischaemic SCID mice. The blood flow of ischaemic hindlimb was evaluated by LDI Doppler laser scanner (Moor Instruments) 30 minutes post surgery and 2 weeks after. The blood flow ratio was defined as the ratio of mean measurement in the foot area of ligated hindlimb to the contralateral unligated hindlimb. Local muscular tissues were harvested following cryosectioning and immunofluorescence staining with human-specific PECAM-1 antibody.

2.2.11 Generation of Dual Seeding Vascular Graft Using *ex vivo* Bioreactor System

The thoracic aorta was excised from the mouse and immediately flushed with saline solution containing 100U heparin to prevent the formation of blood clots. Peri-aortic connective tissue was gently removed with forceps. The aortic graft lumen was flushed with 5 ml 0.075% SDS solution (Severn Biotech Ltd) diluted in PBS and

then soaked in the same solution for 2 hours on an orbital shaker. Then the aorta was flushed and washed in PBS to finish the decellularisation process. The decellularised aorta graft was then fixed in the incubation chamber of a special customised bioreactor circulation system (Zyoxel Ltd, Oxford, UK). The complete setup was maintained in a standard at 37 °C incubator (Figure 2.3).

1×10^6 SMC-CD34-ECs were mixed with 50 μ l of EGM-2 media and seeded inside the decellularised vessel graft via direct injection. 1×10^6 SMCs were mixed with 100 μ l of Matrigel and pipetted onto the graft to form a gel-like wrap. After 12 hours of static incubation to allow the attachment of the cells to the graft, EGM-2 media was delivered through the lumen by a peristaltic pump (Cole-Parmer) with initial flow rate at 5ml/min followed by a stepwise increase to 20 ml/min over 24 hours. The flow rate was then kept at 20 ml/min for 5 days. The circulating and chamber media were changed every other day. The graft was harvested and cryosections was prepared for further analysis.

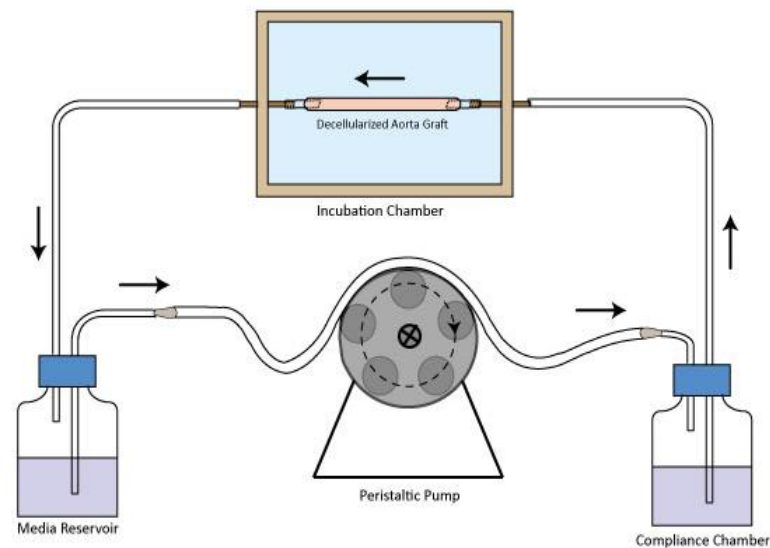


Figure 2.3 Schematic representation of the decellularised graft bioreactor flow circuit

Schematic representation of the decellularised graft bioreactor flow circuit. The decellularised vessel graft is assembled in the incubation chamber. A peristaltic pump is at the upstream of the incubation chamber to provide stable medium perfusion flow. The media reservoir is at the downstream of the incubation chamber. The compliance chamber is to improve the flow regime. The flow direction is indicated by arrows.

2.2.12 Gene Function Analyses

2.2.12.1 Knockdown of target genes through delivery of Short Hairpin RNA (shRNA) Lentiviral Particles

ShRNAs lentiviral particles have been used in this study to perform the knockdown of target genes and to further evaluate the effects caused by gene knockdown. HEK 293T cells were used to produce shRNA lentiviral particles. HEK 293T cells were transfected with the lentiviral vector and the packaging plasmids, pCMV-dR8.2 and pCMV-VSV-G (both from Addgene) using FuGENE HD (Promega). The supernatant containing the lentiviral particles was collected and filtered 48 hours after transfection. The viral titers were determined using the p24 antigen ELISA (Zeptomatrix).

ShRNA E-Cadherin (shECAD, sequence is listed in Table 2.6) (CDH1 MISSION shRNA, SHCLNG_NM_004360, Sigma Aldrich) in lentiviral vector were delivered into PR-SMCs to suppress E-Cadherin expression during reprogramming. The shRNA Non-Targeting (shNT) vector was used as negative control to eliminate off-target effects. 2 day-reprogrammed SMCs were incubated with the shECAD or shNT control (1×10^7 TU/ml) in the complete media supplemented with 10 μ g/ml of Polybrene for 16 hours. Media were changed the next day and the cells were harvested 48 hours after transduction for analysis.

ShRNA HES5 (shHES5, sequence is listed in Table 2.6) (HES5 MISSION shRNA, SHCLNG_XM_371215, Sigma Aldrich) or shRNA JAG1 (shJAG1, sequence is listed in Table 2.6) (JAG1 MISSION shRNA, SHCLNG_NM_000214, Sigma Aldrich) in lentiviral vectors were delivered into SMC-ECs to suppress the expression of HES5 or JAG1 during endothelial differentiation. The shRNA Non-Targeting (shNT) vector was used as negative control to eliminate off-target effects. 3 day-reprogrammed PR-SMCs were incubated with the shHES5/shJAG1 or shNT control (1×10^7 TU/ml) in the complete media supplemented with 10 μ g/ml of Polybrene for 16 hours. Media were changed the next day and the cells were harvested 3 days after transduction for analysis.

Table 2.6 shRNA Sequences

shRNA	Sequence 5'-3'
shECAD	CCGGAGATTGCACCGGTGCGACAAAGCTCGAGCTTTGTGCGACCGGTGCAATCTTTTTTTG
shHES5	CCGGGCTGTCAGCTACCTGAAGCACCTCGAGGTGCTTCAGGTAGCTGACAGCTTTTT
shJAG1	CCGGCCTACTGTGAAACCAATATTACTCGAGTAATATTGGTTTCACAGTAGGTTTTTG

2.2.12.2 Overexpression of HES5 by Plasmid pCMV6-HES5

Plasmid pCMV6-HES5 (RG215311, Origene) containing the full length human HES5 cDNA fragment in a pCMV6-AC vector was used to overexpress HES5 during PR-SMC to the endothelial lineage differentiation. Empty pCMV6 vector was used as the negative control. 3 day-endothelial differentiated PR-SMCs were transfected with pCMV6-HES5 or pCMV6 empty vector using Basic SMC Nucleofector Kit (LONZA) as described in section 2.2.3.3. The expression of the endothelial markers was evaluated at the mRNA and protein levels after 3 days of overexpression.

The sequence of inserted human HES5 Open Reading Form (ORF) is as following:

ATGGCCCCCAGCACTGTGGCCGTGGAGCTGCTCAGCCCCAAAGAGAAAA
ACCGACTGCGGAAGCCGGTGGTGGAGAAGATGCGCCGCGACCGCATCAA
CAGCAGCATCGAGCAGCTGAAGCTGCTGCTGGAGCAGGAGTTCGCGGCA
CCAGCCCAACTCCAAGCTGGAGAAGGCCGACATCCTGGAGATGGCTGTC
AGCTACCTGAAGCACAGCAAAGCCTTCGTCGCCGCCGCCGGCCCCAAGA
GCCTGCACCAGGACTACAGCGAAGGCTACTCGTGGTGCCTGCAGGAGGC
CGTGCAGTTCCTGACGCTCCACGCCGCCAGCGACACGCAGATGAAGCTG
CTGTACCACTTCCAGCGGCCCCCGGCCGCGCCCGCCGCGCCCGCCAAGG
AGCCCAAGGCGCCGGGCGCCGCGCCCCCGCCGCGCTCTCCGCCAAGGC
CACCGCCGCCGCCGCCGCGCGCACCAGCCCGCCTGCGGCCTCTGGCGG
CCCTGG

2.2.12.3 Luciferase Activity Assay

Luciferase activity assay was performed to assess the regulatory role of HES5 or JAG1 on eNOS promoter region during PR-SMC to endothelial lineage differentiation. PR-SMCs were seeded on Collagen IV coated 12-well plate and were differentiated in endothelial-promoting condition for 3 days. 3 day endothelial-differentiated PR-SMCs were transiently co-transfected with 0.33 µg/well reporter plasmid pGL2-eNOS (Plasmid 19297, Addgene) which contains firefly Luciferase under the control of the 1621 bp fragment of human eNOS promoter and 0.1 µg/well reporter plasmid Renilla (Promega) in the presence of 0.16 µg/well pCMV6-HES5 or pCMV6-JAG1 (HG11648-M-N, Sino Biological) or pCMV6 empty vector using Eugene HD (Promega). After 48 hours of incubation, cells were lysed in lysis buffer (Promega). Luciferase and renilla activities were measured with the dual Luciferase Assay System (Promega) with a luminometer (Lumat LB 9507, Berthold Technologies). Renilla activity was used to normalise the transfection efficiency. Relative luciferase activity was defined as the ratio of luciferase activity to Renilla activity with that of empty pCMV6 vector transfected control set as 1.0.

Luciferase assay was also performed to evaluate the effect of JAG1 on HES5 promoter region. 3 day endothelial-differentiated PR-SMCs were co-transfected with luciferase reporter plasmid pGL3 for HES5 promoter and Renilla reporter plasmid in the presence of pCMV6-JAG1 or pCMV6 empty vector.

2.2.12.4 Immobilised Recombinant JAG1 Stimulation

The recombinant human JAG1-FC chimera (R&D systems) was used to stimulate JAG1-induced signals during PR-SMCs to ECs differentiation. Six-well cell culture plates were coated with 50µg/ml of Protein G (life technologies) in PBS at room temperature over night. Then the plates were washed 3 times with PBS and further blocked with 10mg/ml BSA in PBS for 2 hours at room temperature. The plates were washed 3 times with PBS before incubation with 5 µg/ml of recombinant human JAG1-FC chimera in 0.1% BSA/PBS or only IgG as control for 4 hours at room temperature. After washing 3 times with PBS, 3 day-endothelial lineage differentiated PR-SMCs were seeded on the coated plates and maintained under the

same endothelial-promoting conditions. The samples were harvested 2 days after and analysis for endothelial markers was performed.

2.2.13 Differentiation of Mouse iPS cells towards ECs

Mouse iPS cells were seeded on Collagen IV coated flasks in 50 ng/ml VEGF supplemented differentiation media (DM) which consists of α -MEM media with 10% FBS, 0.05 mM β -mercaptoethanol, and 100 u/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in this endothelial-promoting condition for 3, 6, and 9 days when the samples were harvested and analysed for endothelial marker expression at the mRNA and protein levels.

2.2.14 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, Inc.). Data were analysed by two-tailed Student's *t* test or analysis of variance (ANOVA), when *t* test is inappropriate, followed by multiple comparisons with Bonferroni's method. Data were presented as the mean and standard error of the mean (S.E.M.). A value of $p < 0.05$ was considered to be significant.

2.2.15 Illustration Drawing

Illustrations were drawn or adapted with software Adobe Illustrator CS5 (Adobe Systems Inc.)

2.2.16 Study Approval

All animal procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animal.

CHAPTER 3. RESULTS

3.1 Establishment and Optimisation of the Protocol to Derive ECs from Human Vascular SMCs through Reprogramming

3.1.1 Characterisation of the Human Vascular SMC Line

Human Umbilical Artery Smooth Muscle Cells (UASMCs) were used as the human vascular SMCs population in this study. First, it was important to verify that the characteristics of this SMC line comply with commonly recognised SMC features (Owens, Kumar et al. 2004). Under *in vitro* culture conditions, UASMC exhibited an elongated shape and oval nuclei. The cytoplasm was homogeneous and contained few visible inclusions. When confluence was achieved in culture, cells showed the typical SMC hill-and-valley growth pattern (Figure 3.1). Compared to human fibroblasts and Human Umbilical Vein Endothelial Cells (HUVECs), western blot analysis showed that UASMCs expressed a high level of the typical SMC markers including α -SMA, SM22 α , and Calponin and had no expression of EC marker VE-Cadherin (Figure 3.2 A). Immunofluorescence staining of UASMCs showed the representative staining patterns for cytoskeletal and contractile proteins: α -SMA, SM22 α , and Calponin (Figure 3.2 B). To rule out possible EC contamination, UASMCs were immunofluorescence stained to show that they did not express VE-Cadherin.

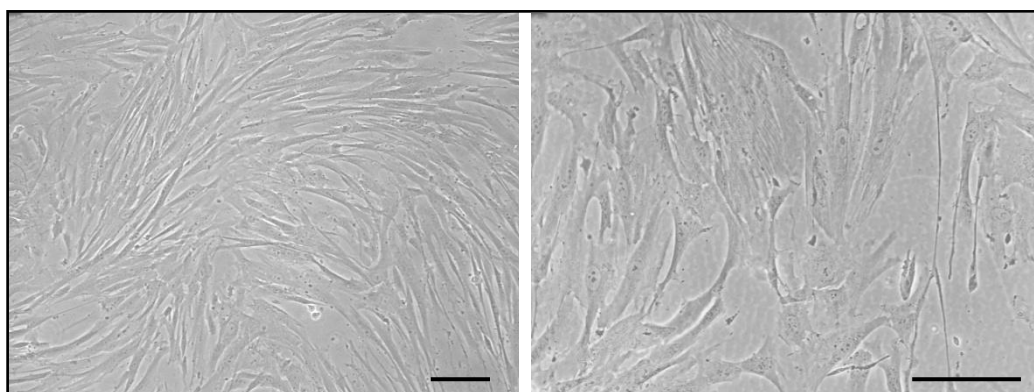


Figure 3.1 Morphology of cultured UASMCs

Representative morphological images showing UASMC displayed an elongated shape, oval nuclei and hill-and-valley growth pattern, which

complied with commonly accepted SMC morphological characteristics. Scale bar: 100 μ m.

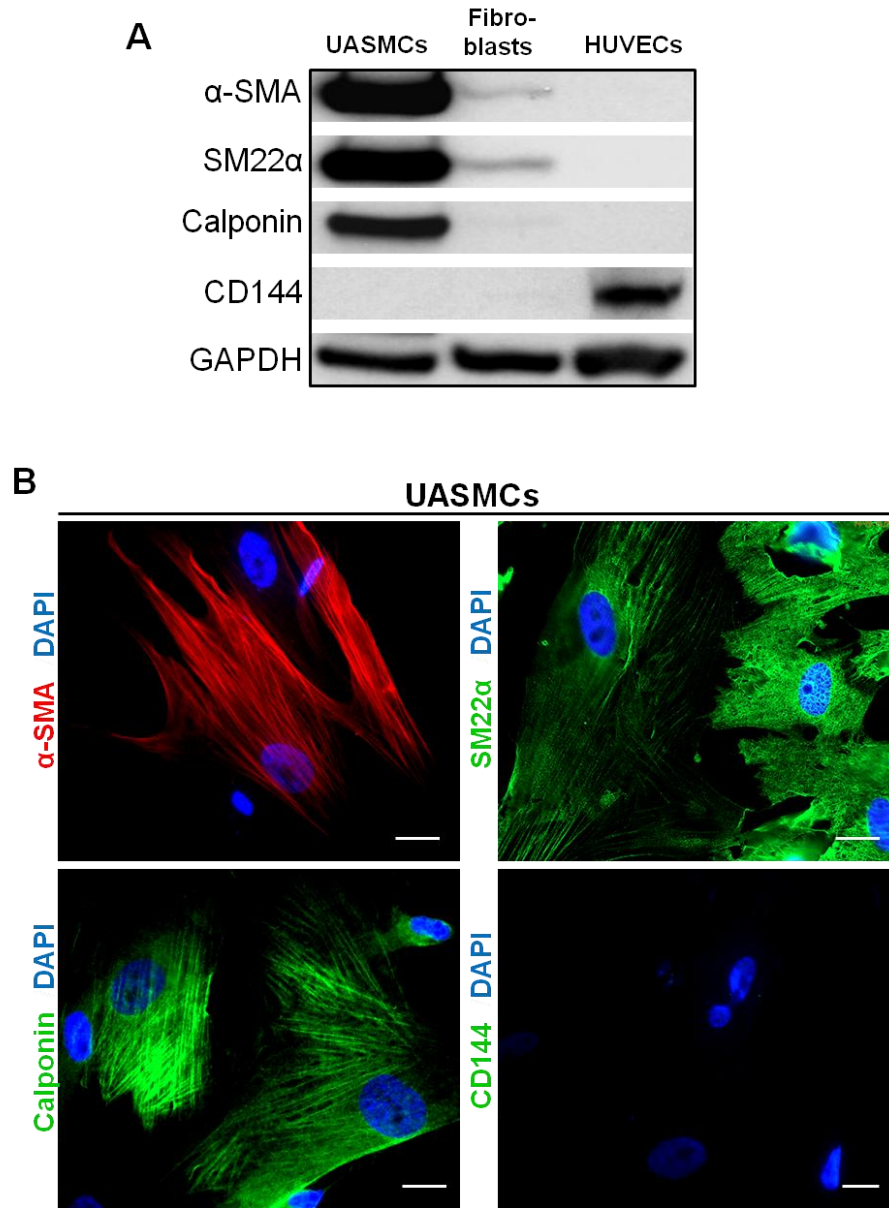


Figure 3.2 Markers expressed by human UASMCs at the protein level

(A) Western blot analysis showed that human UASMCs strongly expressed SMC markers α -SMA, SM22 α , and Calponin compared to human fibroblasts and HUVECs. UASMCs did not express endothelial marker VE-Cadherin compared to HUVECs. **(B)** Representative images of the immunofluorescence staining of UASMCs for SMC markers α -SMA, SM22 α , Calponin and endothelial marker VE-Cadherin. Scale bar: 25 μ m.

3.1.2 Conversion of Human Vascular SMCs to a Vascular Progenitor State through Short Term Reprogramming

To achieve the conversion of SMCs towards an endothelial lineage, a short term (4 days) reprogramming strategy was used. The first step was to induce SMCs back to a partially reprogrammed state by ectopic overexpression of the four transcription factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (Figure 3.3). The reprogramming time is set as 4 day based on following two reasons: (1) genome-wide and proteome-wide analyses during iPS cell generation revealed extensive cellular activities at transcriptional and translational levels from day 0 to day 4, which reflect the alteration of the cell identity- plasticity (Buganim, Faddah et al. 2013); (2) human fibroblasts can be reprogrammed for 4 days to back to a plastic partially iPS cell state that allows further differentiation towards EC or SMC (Margariti, Winkler et al. 2012, Karamariti, Margariti et al. 2013). We named the SMCs after 4 day reprogramming as partially reprogrammed SMCs (PR-SMCs) because a 4 day reprogramming time is much shorter than the general time, 21-28 days, to induce fully reprogrammed iPS cells from human SMCs or human fibroblasts (Takahashi, Tanabe et al. 2007, Lee, Song et al. 2010).

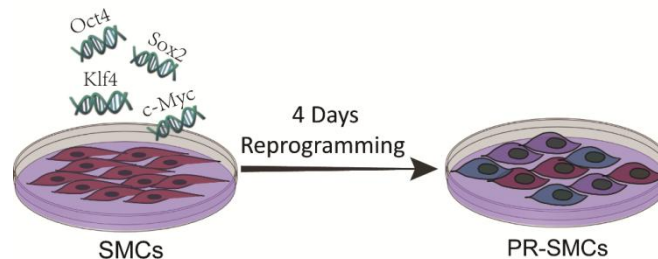


Figure 3.3 Schematic protocol of the short term reprogramming of SMCs towards the partially reprogrammed state.

SMCs were transfected with lentiviral vector containing the four reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* and then maintained in the reprogramming media for 4 days to acquire the partially reprogrammed state.

SMCs were transfected with the lentivirus TetO-OSKM encoding OCT4, SOX2, KLF4 and c-MYC or the empty vector as control and then maintained under the reprogramming conditions for 4 days. Transduced SMCs strongly overexpressed the corresponding mRNAs and proteins (Figure 3.4). After 4 days of reprogramming, PR-SMCs displayed distinct morphological changes compared to the empty vector transfected control group (Figure 3.5). PR-SMCs also lost the expression of typical SMC markers which can be seen in real-time PCR results (Figure 3.6). Notably, an upregulation of CD34, a vascular progenitor marker, was observed in the PR-SMCs population at the mRNA and the protein level at this point (Figure 3.7). Other commonly used vascular progenitor markers including KDR, C-KIT, CD133 showed no significant upregulation (Figure 3.8). Previous studies have shown that CD34-positive vascular progenitors are able to give rise to both endothelial- and smooth muscle-like cells (Ferreira, Gerecht et al. 2007, Kurian, Sancho-Martinez et al. 2013). Thus, 4 day reprogramming with *OCT4*, *SOX2*, *KLF4* and *c-MYC* erases the original cell identity of SMCs and reverses them to a vascular progenitor state.

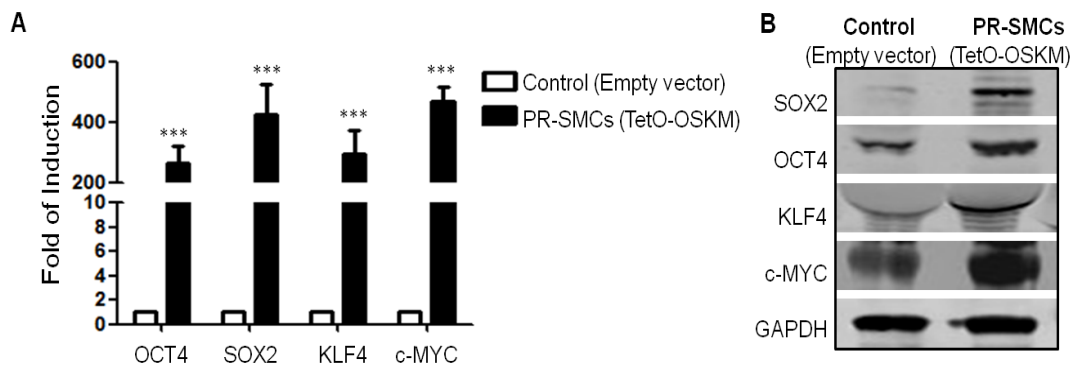


Figure 3.4 The overexpression of the four reprogramming factors after 4 day reprogramming

(A) Real-time PCR showed the successful overexpression of the four reprogramming factors OCT4, SOX2, KLF4 and c-MYC at the mRNA level of PR-SMCs. SMCs transfected with empty lentiviral vector and kept under identical reprogramming conditions were used as the control group. (***) $p < 0.001$ by Student's *t* test, $n=4$) **(B)** Western blot analysis confirmed the overexpression of the transcription factors SOX2, OCT4, KLF4 and c-MYC at the protein level of PR-SMCs.

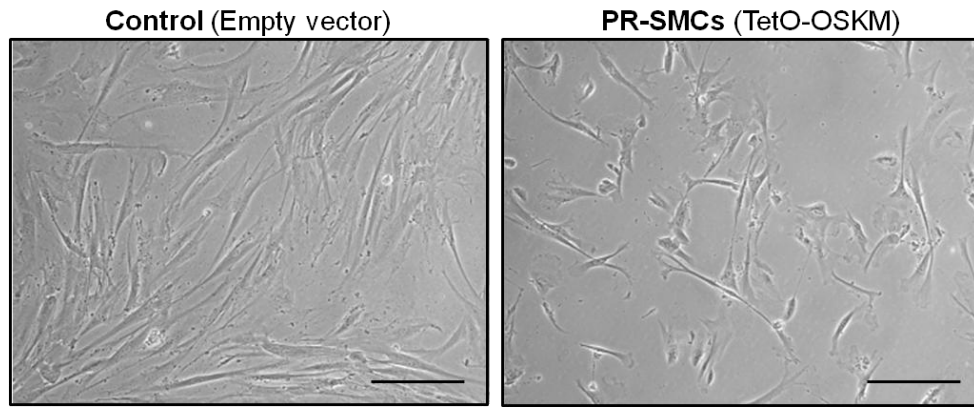


Figure 3.5 Cell morphology of PR-SMCs

Representative image of PR-SMCs displayed a distinct morphology compared to the control group. SMCs transfected with empty lentiviral vector and maintained under the same reprogramming conditions were used as the control group. Scale bar: 100 μ m.

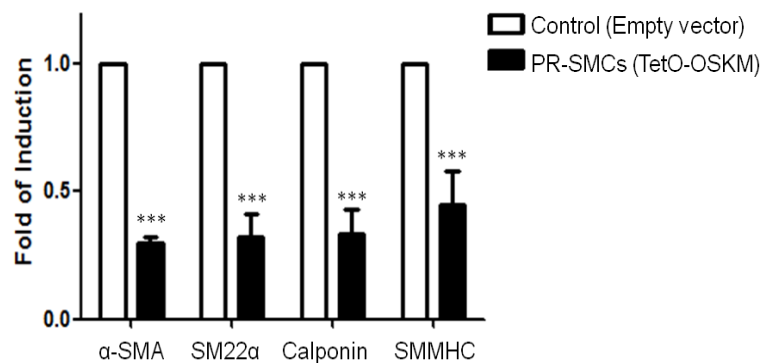


Figure 3.6 The downregulation of SMC marker expression by PR-SMCs

Real-time PCR analysis showed an overall suppression of typical SMC markers in PR-SMCs compared to control cells. SMCs started to lose their original identities after 4 day reprogramming (** $p < 0.001$ by Student's t test, $n=3$). SMCs transfected with empty lentiviral vector and maintained under the same reprogramming conditions were used as the control group.

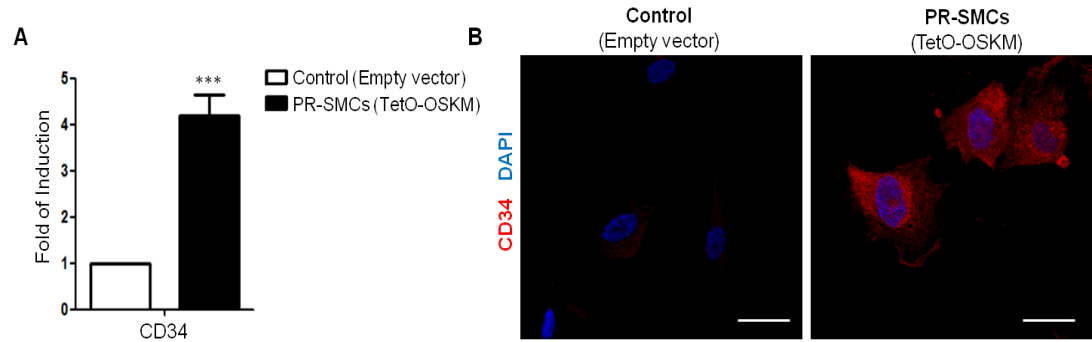


Figure 3.7 Upregulation of CD34 within a PR-SMCs population after 4 day reprogramming

(A) Real-time PCR showed an upregulation in transcription of vascular progenitor marker CD34 by PR-SMCs. (** $p < 0.001$ by Student's t test, $n=4$) (B) Representative images of immunofluorescence staining demonstrated the expression of CD34 in PR-SMCs. SMCs transfected with empty lentiviral vector and treated with the same reprogramming condition were used as the control group. Scale bar: 25 μm .

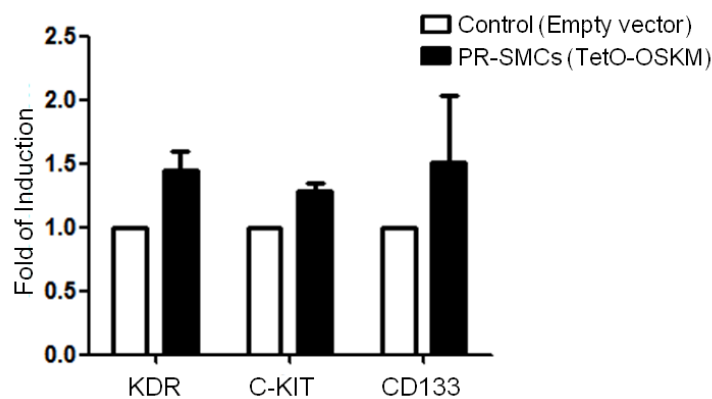


Figure 3.8 Expression of other vascular progenitor markers in PR-SMCs after 4 day reprogramming

Real-time PCR showed no significant difference of other commonly used vascular progenitor markers including KDR, C-KIT, and CD133 in PR-SMCs after 4 day reprogramming. (Student's t test, $n=3$). SMCs transfected with empty lentiviral vector and treated with the same reprogramming condition were used as the control group.

To further evaluate the upregulation of CD34 expression along reprogramming, real-time PCR analysis was performed on samples from different time points during reprogramming. An increase of CD34 was observed along reprogramming that reached a significant level of upregulation at day 4 (Figure 3.9). Although there seemed to be a further CD34 upregulation when the reprogramming time prolonged to 6 days, we stayed with the 4 day reprogramming because the upregulation level was not significant and the longer reprogramming time with pluripotency factors always lead to higher tumour-forming risk.

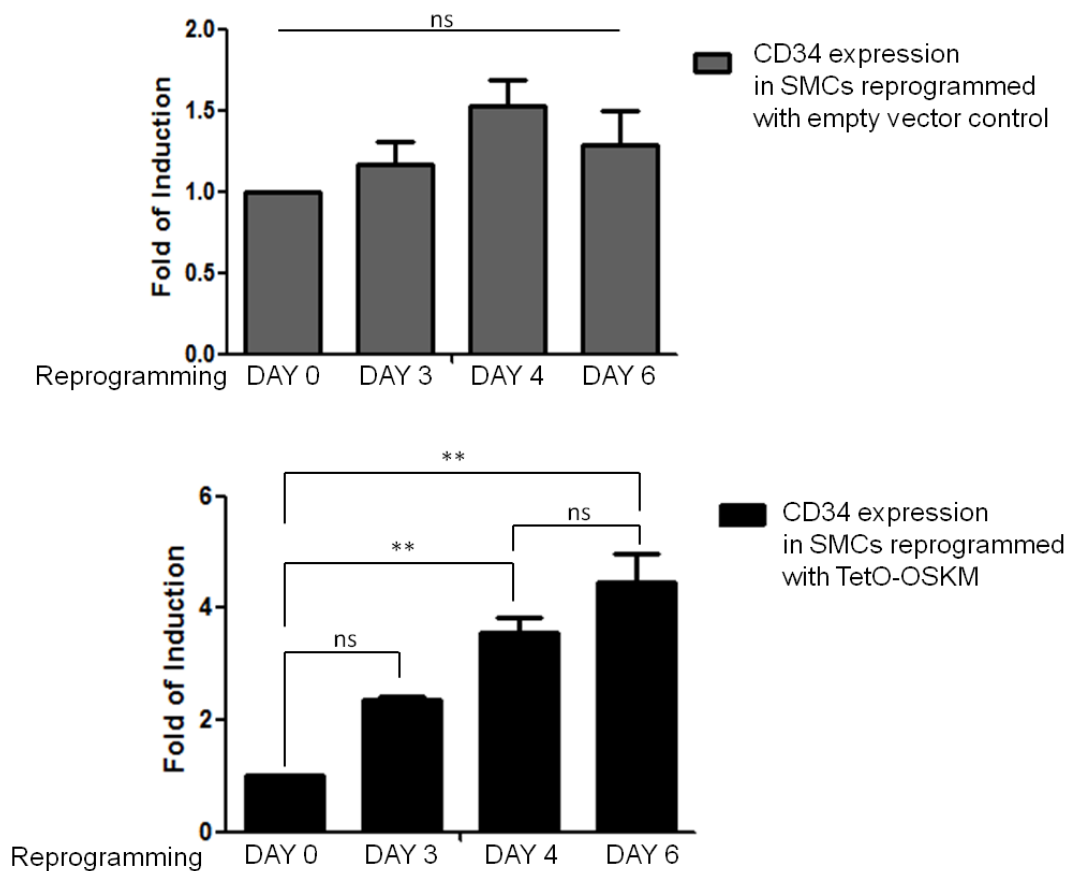


Figure 3.9 The expression of CD34 in reprogrammed SMC at different time point

Real-time PCR was performed to evaluate the CD34 expression at different time point during SMCs reprogramming. CD34 expression in SMC transfected with empty lentiviral vector has no significant change along with reprogramming. CD34 expression in SMC transfected with TetO-OSKM increases along with reprogramming. (** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA followed by multiple comparisons with Bonferroni's method, $n=3$)

3.1.3 Partially Reprogrammed SMCs (PR-SMCs) Display the Potential to Differentiate towards an Endothelial Lineage

To drive the differentiation of vascular progenitor state PR-SMCs towards an endothelial lineage, cells were treated using endothelial-promoting culture condition based on established protocols used for the differentiation of pluripotent cells or progenitor cells to ECs. In general, substrate Collagen IV and growth factor VEGF are important triggers for endothelial lineage induction (Hirashima, Kataoka et al. 1999, Zeng, Xiao et al. 2006, Levenberg, Ferreira et al. 2010, Rufaihah, Huang et al. 2011). A Collagen IV substrate provides a basal lamina-like structure *in vitro* to support the differentiation of stem cells towards an endothelial lineage (Schenke-Layland, Angelis et al. 2007). VEGF is an essential growth factor for endothelial development during embryogenesis and a potent stimulus to induce stem cell differentiation into an endothelial lineage *in vitro* (Coultas, Chawengsaksophak et al. 2005, Nourse, Halpin et al. 2010). Thus, to induce endothelial differentiation, PR-SMCs were seeded on Collagen IV coated petri-dishes and maintained in EGM-2 media supplemented with 25 ng/ml VEGF (Figure 3.10). Under these conditions, SMC-derived ECs (SMC-ECs) were generated and further analysis was performed following 6 days of differentiation. SMCs transfected with the empty TetO lentiviral vector underwent the identical reprogramming and differentiation process and were used as the control group.

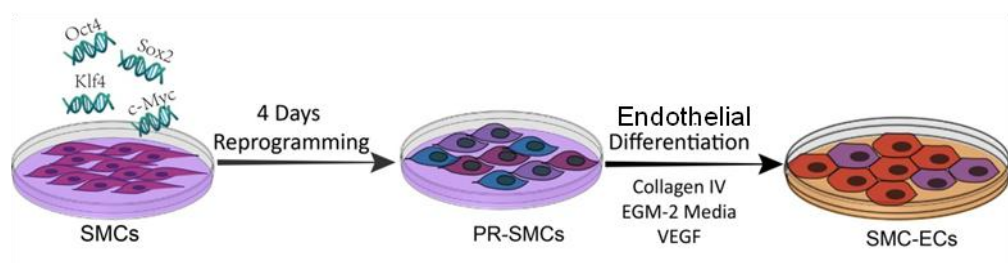


Figure 3.10 Schematic protocol of the further differentiation of PR-SMCs towards an endothelial lineage through culturing under endothelial-promoting conditions

To drive the endothelial differentiation, PR-SMCs were seeded on Collagen IV coated petri-dishes and maintained in EGM-2 media supplemented with 25 ng/ml VEGF.

After 6 days of endothelial-promoting stimulation, SMC-ECs demonstrated altered morphology, appearing shorter and rounder than empty vector transfected control cells (Figure 3.11). At this point, real-time PCR analysis showed that a panel of typical endothelial markers including PECAM-1, VE-Cadherin, eNOS, VWF, and Claudin 5 (CLDN5) was significantly upregulated at the mRNA level in the SMC-ECs (Figure 3.12 A). Western blot analysis confirmed the expression of specific endothelial markers PECAM-1 and VE-Cadherin by SMC-ECs at the protein level (Figure 3.12 B). Moreover, immunofluorescence staining showed the typical cell membrane staining pattern of PECAM-1 for SMC-ECs (Figure 3.13). Flow cytometric analysis further confirmed the increase of endothelial marker PECAM-1 expression at 6.57% and CD34 at 37.72% compared to the empty vector control cells (Figure 3.14). The reprogramming factors were significantly downregulated in the differentiated SMC-ECs population compared to PR-SMCs (Figure 3.15). Altogether, these results indicate that PR-SMCs display the potential to commit to an endothelial lineage in response to endothelial-promoting stimuli.

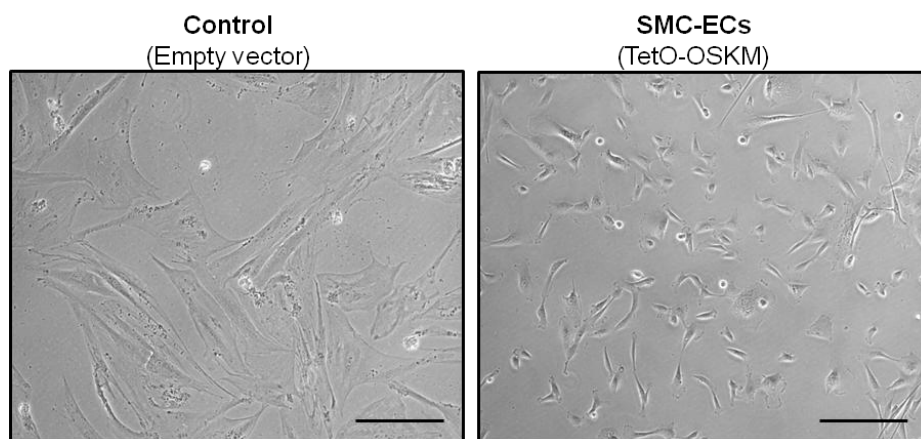


Figure 3.11 Cell morphology of SMC-ECs

SMCs transfected with an empty lentiviral vector that underwent the same reprogramming and differentiation protocol were used as control group.

SMC-ECs exhibited profound morphological changes when compared to this control group. Scale bar: 100 μ m.

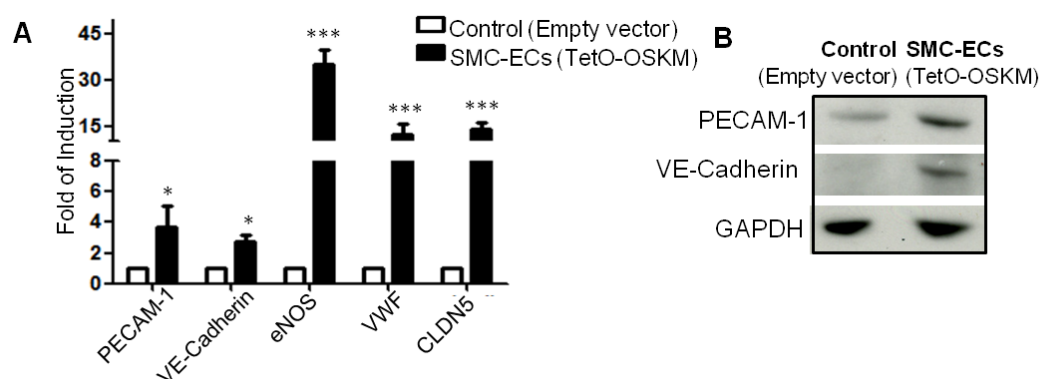


Figure 3.12 Endothelial markers identified in the SMC-ECs population

(A) Real-time PCR results revealed an increase in endothelial markers including PECAM-1, VE-Cadherin, eNOS, VWF, and CLDN5 at the mRNA level. (* $p < 0.05$, *** $p < 0.001$ by Student's t test, $n = 5$) **(B)** Western blot analysis showed the expression of PECAM-1 and VE-Cadherin at the protein level of SMC-ECs. SMCs transfected with empty lentiviral vector that underwent the same reprogramming and differentiation protocol were used as the control group.

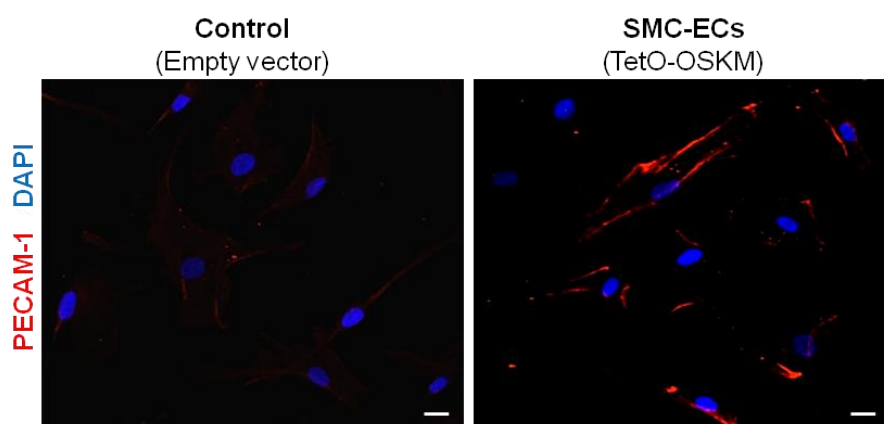


Figure 3.13 Immunofluorescence staining of SMC-ECs for endothelial markers

Representative images of the immunofluorescence staining for endothelial markers PECAM-1 of SMC-ECs. SMC-ECs stained with PECAM-1 showed

the typical membrane staining pattern. SMCs transfected with empty lentiviral vector that underwent the same reprogramming and differentiation protocol were used as the control group. Scale bar: 25 μ m.

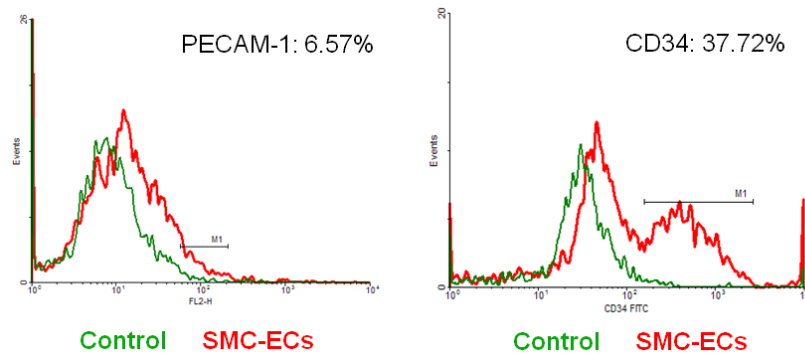


Figure 3.14 Flow cytometric analysis of SMC-ECs for endothelial markers

Flow cytometric histograms demonstrated the increase of the expression of PECAM-1 for 6.57% and CD34 for 37.72% in SMC-ECs compared to the control group. SMCs transfected with empty lentiviral vector that underwent the same reprogramming and differentiation protocol were used as the control group. The gate M1 region was set with $<1\%$ of the peak of the control cells inside the gate's left edge.

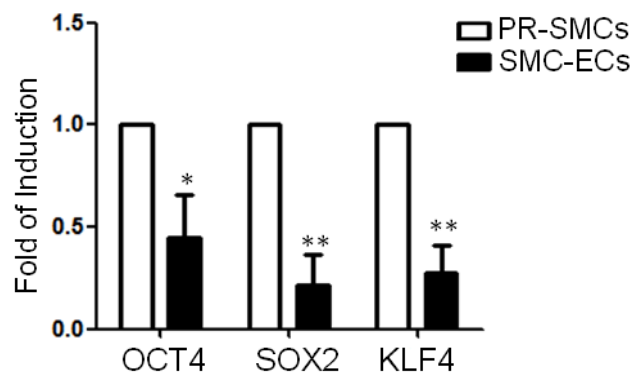


Figure 3.15 Downregulation of transfected reprogramming factors in the SMC-ECs population

Real-time PCR results showed a decrease of ectopic overexpressed reprogramming factors including OCT4, SOX2 and KLF4 in differentiated SMC-ECs compared to PR-SMCs. (* $p<0.05$, ** $p<0.01$ by Student's t test, $n=3$)

3.1.4 Verifying the Protocol for Derivation of ECs from SMCs Using a Different Gene Delivery Method to Overexpress the Four Reprogramming Factors

To verify that the four reprogramming factors were the main force that initiated SMC to endothelial lineage conversion, we employed an alternative method to ectopically overexpress *OCT4*, *SOX2*, *KLF4*, and *c-MYC* in SMCs. SMCs were transfected with a linearised pCAG2LMKOSimO (pCAG-OSKM) plasmid encoding the four factors or with an empty plasmid as control and then treated using an identical protocol of reprogramming and differentiation to that described above. SMCs transfected with pCAG-OSKM plasmid successfully overexpressed the four reprogramming factors (Figure 3.16). After 4 days of reprogramming, PR-SMCs generated using the pCAG-OSKM plasmid displayed a changed morphology compared to empty vector control cells (Figure 3.17). Real-time PCR analysis revealed the upregulation of CD34 at this point (Figure 3.18). The induction of typical endothelial markers was observed at both the mRNA and the protein level after 6 days endothelial-promoting differentiation (Figure 3.19). The above results further validated our finding that SMCs are amenable to conversion into an endothelial lineage through introduction of the four reprogramming factors and further endothelial-promoting stimulation.

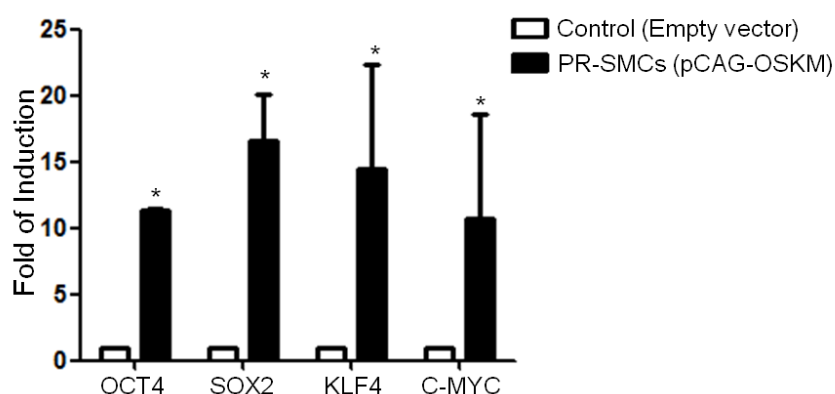


Figure 3.16 The overexpression of four reprogramming factors after pCAG-OSKM transfection

After 4 day reprogramming following pCAG-OSKM plasmid or empty plasmid vector transfection, real-time PCR detected the overexpression of OCT4, SOX2, KLF4 and c-MYC in PR-SMCs compared to the control cells. (* $p < 0.05$ by Student's *t* test, $n = 3$). SMCs transfected with empty pCAG vector that underwent the same reprogramming protocol were used as the control group.

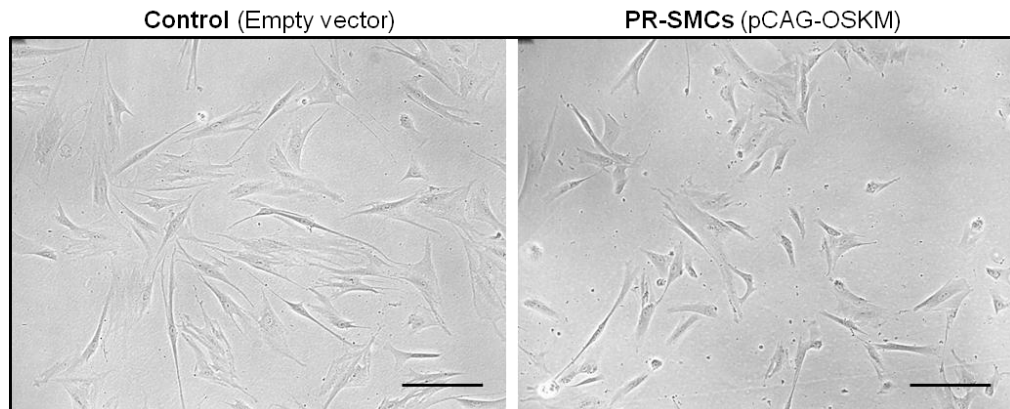


Figure 3.17 Cell morphology of PR-SMCs generated with plasmid pCAG-OSKM

PR-SMCs generated with plasmid pCAG-OSKM displayed partially changed morphology, which were shorter and smaller, compared to the cells transfected with empty plasmid vector. Scale bar: 100 μm .

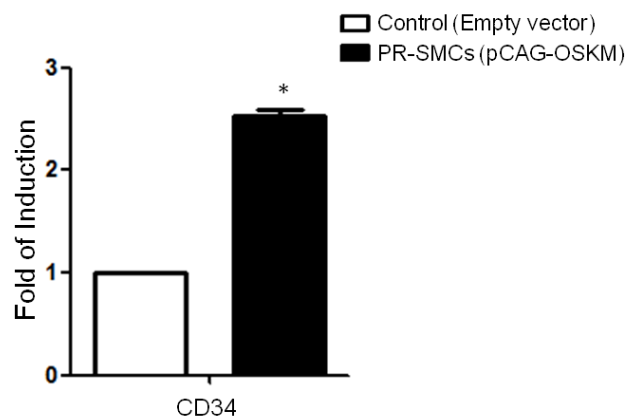


Figure 3.18 PR-SMCs generated using pCAG-OSKM had increased CD34 expression after 4 day reprogramming

Real-time PCR revealed an upregulation of CD34 in PR-SMCs generated with pCAG-OSKM. (* $p < 0.05$ by Student's *t* test, $n = 3$). SMCs transfected with

empty pCAG vector that underwent the same reprogramming protocol were used as the control group.

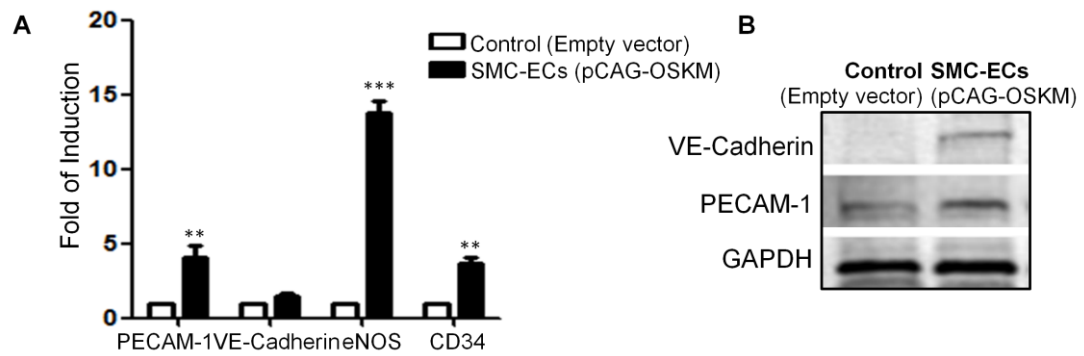


Figure 3.19 Endothelial marker expression in SMC-ECs generated using the pCAG-OSKM

(A) After 6 days of endothelial-promoting differentiation, real-time PCR analysis confirmed that endothelial markers PECAM-1, eNOS, CD34 were upregulated in SMC-ECs at the mRNA level compared to the control cells. (** $p < 0.01$, *** $p < 0.001$ by Student's t test, $n=3$) **(B)** Western blot analysis demonstrated the induction of VE-Cadherin and PECAM-1 expression of SMC-ECs. SMCs transfected with empty pCAG vector that underwent the same reprogramming and differentiation protocol were used as the control group.

3.1.5 CD34-Positive Cells Selected from PR-SMCs Give Rise to a More Homogenous Endothelial Population

From the above results, the emergence of CD34-positive cells among PR-SMCs was observed. Previous studies have shown that CD34 can be considered as a marker of vascular progenitor cells that can give rise to ECs (Ferreira, Gerecht et al. 2007, Kurian, Sancho-Martinez et al. 2013). CD34 has also been identified as a marker of endothelial progenitor cells with the capacity to facilitate neovascularisation (Mackie and Losordo 2011, Fadini, Losordo et al. 2012). The emergence of a CD34-positive population after short term reprogramming with *OCT4*, *SOX2*, *KLF4*, and *c-MYC* indicated the potential induction of a vascular progenitor state from SMCs. Therefore, it was of great interest to test whether CD34-positive cells selected from the heterogeneous PR-SMCs population had enhanced endothelial differentiation capacity. CD34-positive cells were sorted from PR-SMCs using anti-CD34 antibody-coupled magnetic microbeads, seeded on Collagen IV coated petri-dishes and cultured in the same endothelial-promoting 25 ng/ml VEGF-supplemented EGM-2 media as in previous studies shown above (Figure 3.20).

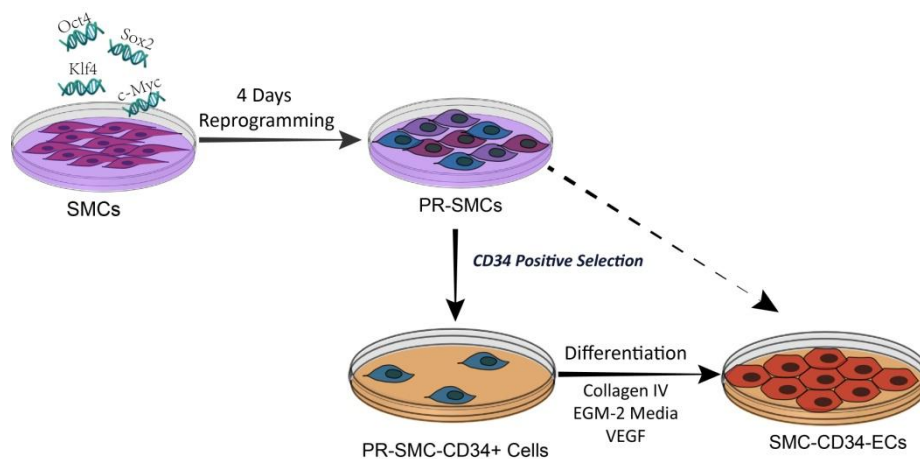


Figure 3.20 Schematic protocol of selecting CD34-positive cells from PR-SMCs and further differentiating towards endothelial lineage

CD34-positive cells were selected from PR-SMCs population and were kept in endothelial-promoting culture condition to induce endothelial differentiation.

After 10 days of differentiation, CD34-positive cells formed a homogeneous endothelial-like monolayer (Figure 3.21). CD34-positive cells selected from the 4 days reprogrammed cells with a control vector were of too little amount and which could not survive. Therefore, the original SMCs population was used as control group. CD34-positive cells could be selected from SMCs, however, these cells only gave rise to SMC-like cells after 2 weeks in the same endothelial-promoting conditions.

Real-time PCR analysis showed a significant induction of a full panel of endothelial markers including VEGFR2, PECAM-1, VE-Cadherin, eNOS, VWF and the suppression of SMC markers including α -SMA, SM22 α , Calponin, SM-MHC at the mRNA level in SMC-derived CD34-positive cells converted ECs (SMC-CD34-ECs) compared to SMCs (Figure 3.22). Transcriptome sequencing (RNA-Seq) was performed on the cell populations at different stages of the SMC to EC conversion including SMCs, PR-SMCs, SMC-CD34-ECs and HUVEC. These genome-wide analyses showed the global gene expression of SMC-CD34-ECs displayed an overall shift towards the human endothelial cell line (Figure 3.23). The upregulation of a cluster of endothelial enriched genes and downregulation of a cluster of SMC genes was detected at the transcriptome level of SMC-CD34-ECs (Figure 3.24). Gene ontology analysis of the upregulated gene profile of SMC-CD34-ECs indicated the enrichment of genes for immune response and cell adhesion related biological processes, which are usually identified in ECs (Figure 3.25).

At the protein level, western blot analysis validated the strong induction of endothelial markers including VE-Cadherin, PECAM-1, and eNOS (Figure 3.26). Flow cytometric analysis revealed the elevated expression of endothelial markers PECAM-1, VE-Cadherin, and VEGFR2 at 30.19%, 32.56% and 40.19% respectively in SMC-CD34-ECs population compared to SMCs (Figure 3.27). Furthermore, immunofluorescence staining displayed the typical junctional expression pattern of PECAM-1 and VE-Cadherin and positive staining for VEGFR2 of SMC-CD34-ECs (Figure 3.28). Based on the above findings, CD34-positive PR-SMCs represented a more optimised cell population for endothelial lineage differentiation compared to the heterogeneous PR-SMC population.

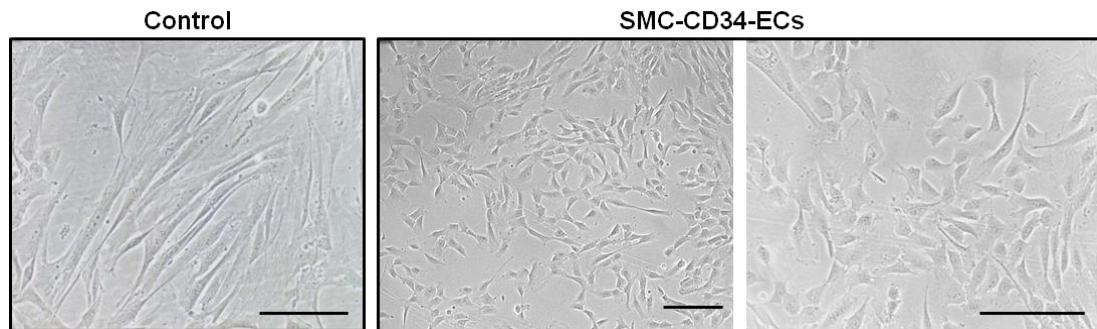


Figure 3.21 Cell morphology of SMC-CD34-ECs

SMC-CD34-ECs displayed a homogeneous endothelial-like morphology compared to SMCs. Scale bar: 100 μ m.

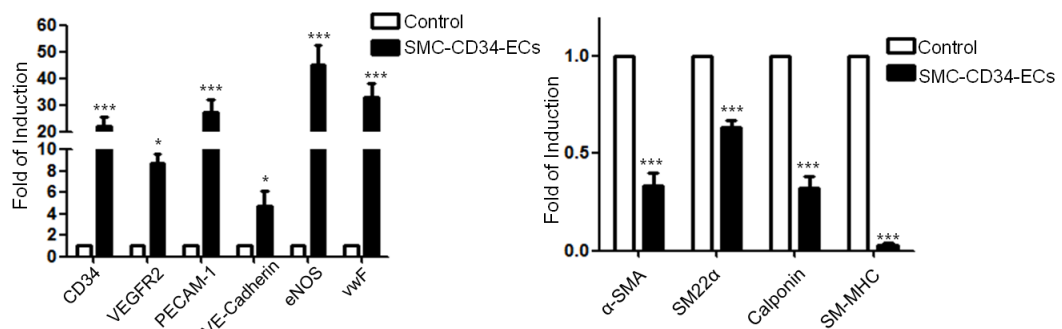


Figure 3.22 The expression of endothelial and smooth muscle markers in SMC-CD34-ECs

Real-time PCR results showed a strong induction of endothelial markers including VEGFR2, PECAM-1, VE-Cadherin, eNOS, VWF and a significant suppression of smooth muscle markers α -SMA, SM22 α , Calponin and SM-MHC in SMC-CD34-ECs compared to SMC control. (* p <0.05, *** p <0.001 by Student's t test, n =4)

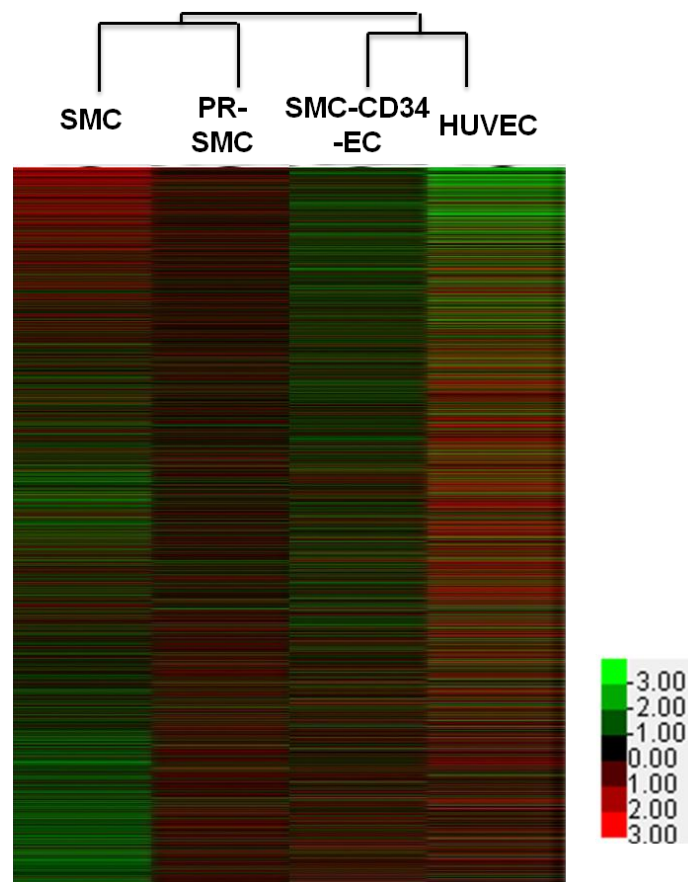


Figure 3.23 Global gene expression of SMCs, PR-SMCs, SMC-CD34-ECs and HUVECs

Heat map shows the hierarchical clustering analysis of global gene expression from RNA-Seq results of SMCs, PR-SMCs, SMC-CD34-ECs and HUVECs. Colour bar indicates gene expression in scale. Genes expressed at low level are indicated with colour green while genes expressed at high level are indicated with colour red. The global gene expression pattern showed a shift of the overall gene expression from SMC towards ECs.

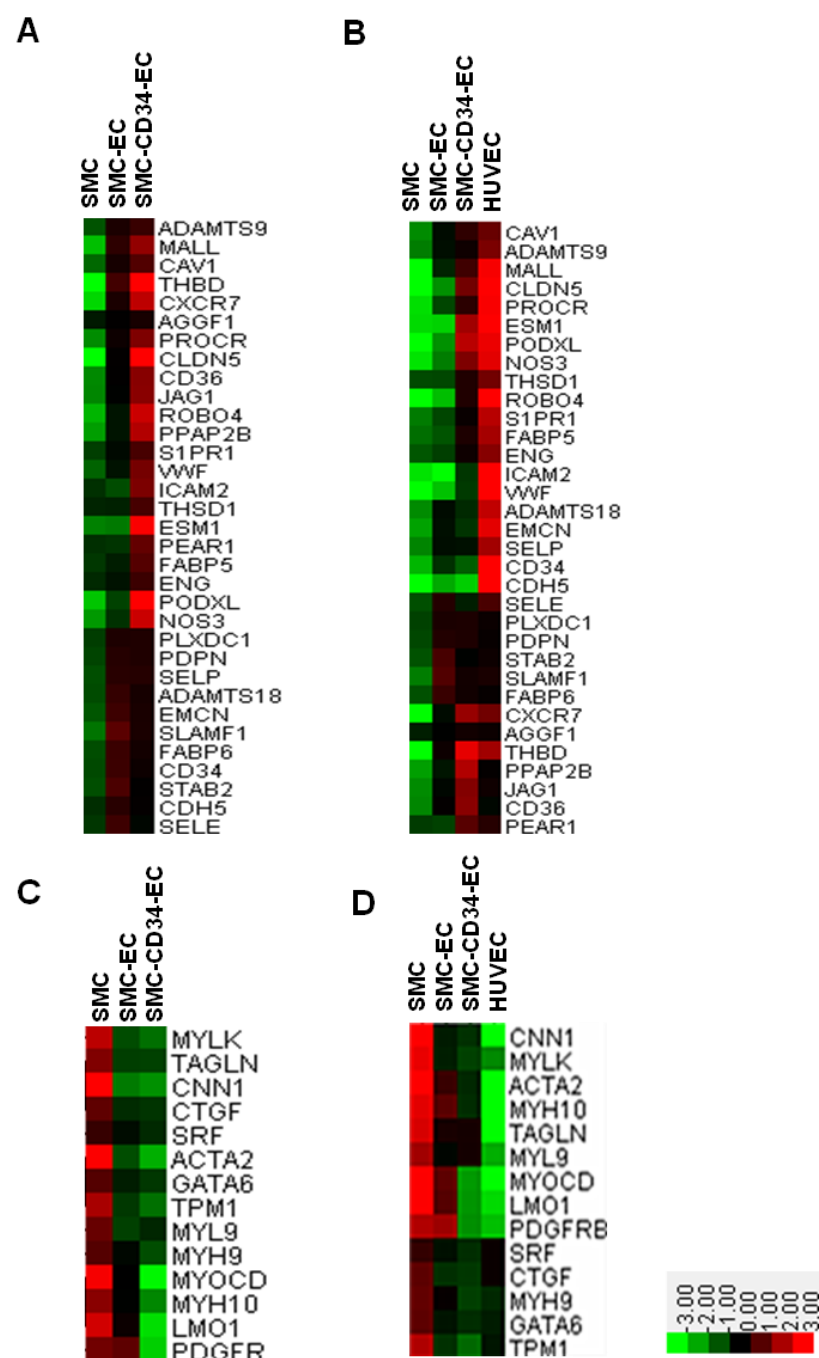


Figure 3.24 Heat map of EC and SMC enriched gene expression changes among SMC, SMC-ECs, SMC-CD34-ECs and HUVECs

Based on RNA-Seq results, here shows the heat map of the changes of commonly recognised **(A, B)** EC and **(C, D)** SMC enriched genes expression among SMC, SMC-ECs, SMC-CD34-ECs, and HUVECs. Overall, EC enriched genes went up and SMC enriched genes went down in SMC-ECs and SMC-CD34-ECs compared to SMCs. Colour bar indicates gene expression in scale.

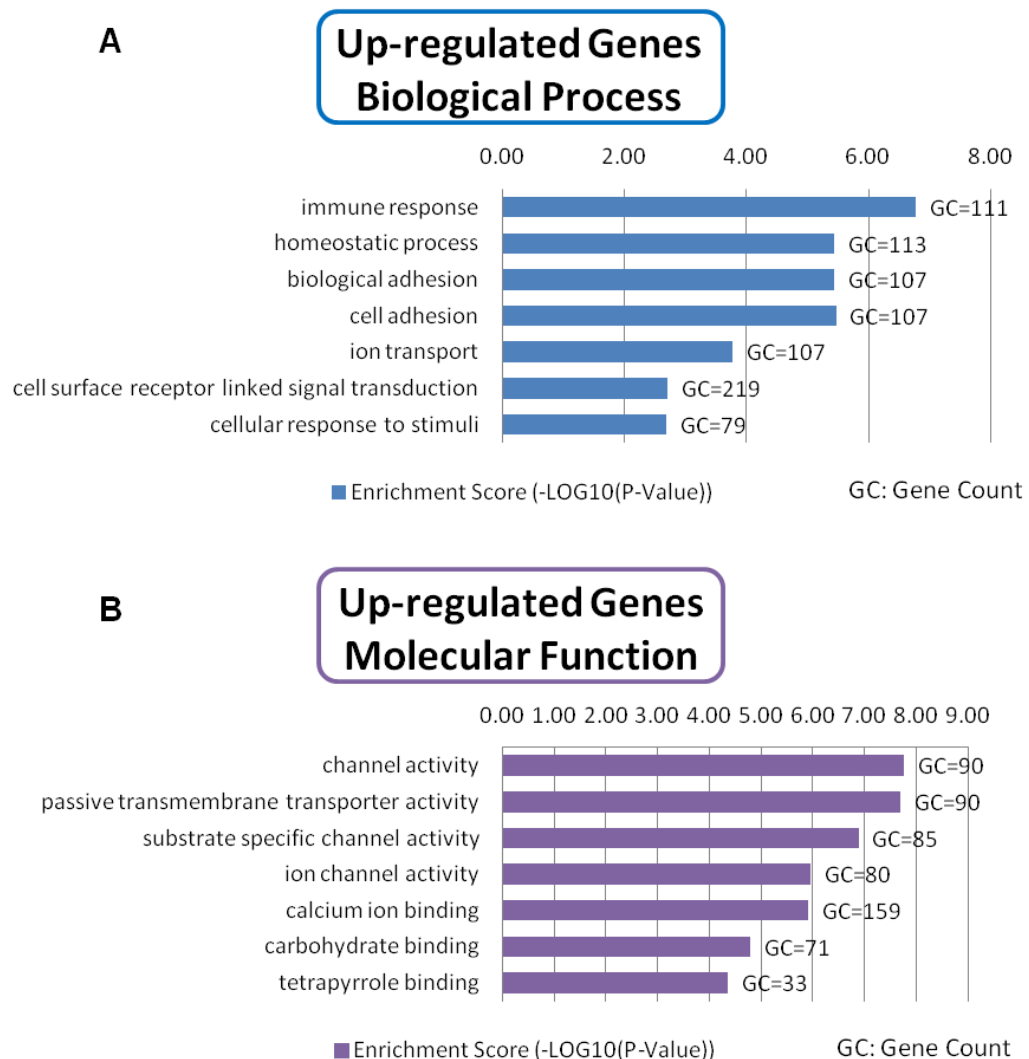


Figure 3.25 Gene ontology analysis of the upregulated genes between SMC-CD34-ECs and SMCs

Gene ontology (GO) analysis was performed on the upregulated genes of SMC-CD34-ECs compared to SMCs from the RNA-Seq data. An online DAVID bioinformatics resource 6.7 functional annotation tool suite was used to carry out the GO analysis. Enrichment in gene ontology of **(A)** biological process and **(B)** molecular function were revealed. Significance of enrichment was evaluated by p-value which is calculated by the software. The closer of p-value to zero, the more significant of the GO term annotated to the genes.

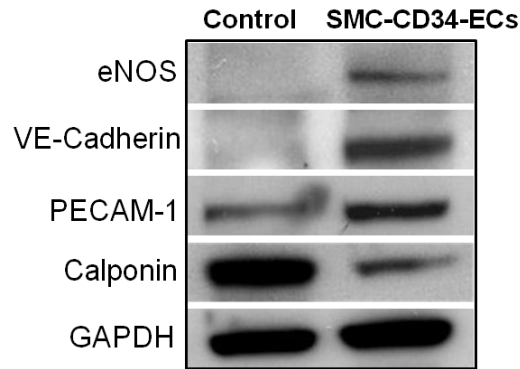


Figure 3.26 Western blot analysis of the expression of EC and SMC markers by SMC-CD34-ECs

Western blot analysis confirmed the upregulation of endothelial markers eNOS, VE-Cadherin, PECAM-1 and the downregulation of SMC marker Calponin in SMC-CD34-ECs at the protein level compared to SMCs.

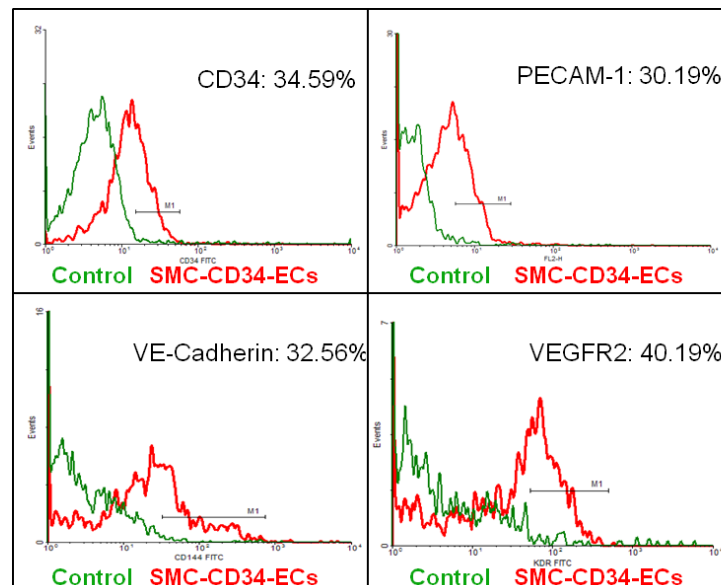


Figure 3.27 Flow cytometric analysis of endothelial markers of SMC-CD34-ECs

Flow cytometric histograms evaluated the increase of the progenitor marker CD34 and endothelial markers PECAM-1, VE-Cadherin, VEGFR2 at 34.59%, 30.19%, 32.56% and 40.19% respectively of SMC-CD34-ECs compared to SMCs. The marker region of gate M1 was set with <1% of the peak of the control cells inside the gate's left edge.

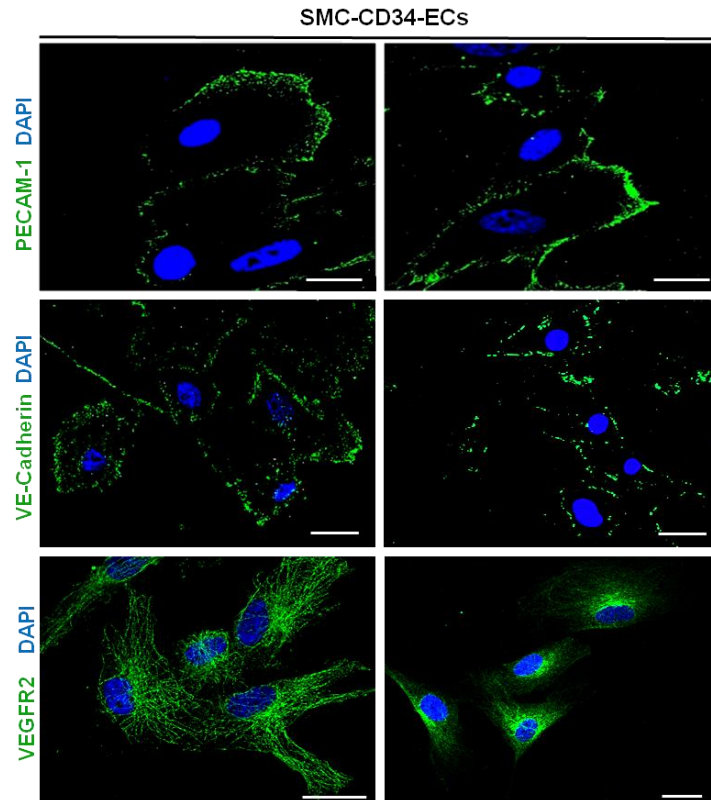


Figure 3.28 Immunofluorescence staining for endothelial markers in SMC-CD34-ECs

Immunofluorescence staining of SMC-CD34-ECs revealed the typical junctional staining pattern of PECAM-1, VE-Cadherin and the positive staining for VEGFR2. Scale bar: 25 μ m.

3.1.6 Conclusion

In this first part of the work, an optimised protocol for the conversion of human vascular SMC towards an endothelial lineage has been established.

First, we reprogrammed the human vascular SMCs with the four transcription factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* for 4 days to generate partially reprogrammed SMCs (PR-SMCs). At this stage, PR-SMCs expressed an upregulated level of a vascular progenitor marker: CD34. SMCs were converted to a vascular progenitor state by short term reprogramming.

Next, the PR-SMCs were treated with endothelial-promoting conditions: the combination of Collagen IV substrate and VEGF supplemented EGM-2 media. After 6 days, the cells expressed a panel of endothelial markers at both the mRNA and the protein level. Thus, ECs were generated from the SMCs after 4 days of reprogramming and 6 days of differentiation.

To further improve the protocol, CD34-positive cells were selected from the PR-SMCs and then maintained in the endothelial-promoting conditions. CD34-positive PR-SMCs exhibited an enhanced capacity to give rise to a homogeneous endothelial population with a complete repertoire of endothelial features.

Taken together, the feasibility of the conversion of human SMC to an endothelial lineage has been proven. We presented a two step protocol consisting first of reprogramming the SMCs to CD34-positive vascular progenitors and subsequently differentiating the cells towards the endothelial lineage.

3.2 Functional Characterisation of the SMC-derived ECs

3.2.1 SMC-converted ECs Display Endothelial Functionality both *In Vitro* and *In Vivo*

Following the gene and protein expression profile characterisation of SMC-derived ECs, it was of great interest to evaluate the endothelial related function of the converted endothelial population.

The ability to produce vasodilator Nitric Oxide (NO) is one of the critical characteristics of functional ECs. The level of cellular NO was measured by a specific probe: fluorescein amine methyl ester (FA-OMe). Non-fluorescent FA-OMe transforms into intense fluorescent deamination product, da-FA-OMe, when sensing free NO in living cells (Shiue, Chen et al. 2012). By using this method, NO produced by SMC-ECs was detected within the cytoplasm while empty vector control cells did not exhibit the ability to produce NO (Figure 3.29).

Another important endothelial function is to regulate lipid metabolism, which can be reflected in their ability to take up acetylated low density lipoprotein (ac-LDL) (Voyta, Via et al. 1984). SMC-CD34-ECs could effectively take up fluorescently labelled acetylated low density lipoprotein (ac-LDL), which demonstrated that their lipid metabolism regulatory ability resembled that of mature ECs (Figure 3.30).

To assess the vasculogenic and angiogenic potential of the cells, *in vitro* Matrigel tube formation assay was performed. Matrigel is a type of biologically active reconstituted matrix extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in all kinds of ECM proteins present in the basement membrane (Kleinman and Martin 2005). This assay is a quick method to evaluate the ability of ECs to form three dimensional tubular structures on *in vitro* ECM gel that mimics the *in vivo* cellular basement membrane environment. SMC-CD34-ECs or SMCs suspended in VEGF supplemented EGM-2 media were seeded onto Matrigel and maintained in a 37 °C incubator. SMC-CD34-ECs clearly aggregated into vessel-like tube structures on Matrigel after 6 hours of incubation (Figure 3.31 A).

Quantification of the total tube length revealed that converted ECs exhibited a much stronger tube-forming capacity than the SMCs control (Figure **3.31 B**). Moreover, the tube-forming cells of the SMC-CD34-ECs group also stained positively for endothelial marker VE-Cadherin (Figure **3.31 C**).

Subsequently, a subcutaneous Matrigel plug assay in mice was performed to further assess the angiogenic ability of SMC-converted ECs *in vivo*. 5×10^5 SMC-CD34-ECs or SMCs were mixed with 50 μ l EGM-2 media and 250 μ l Matrigel. The cell mixture was injected subcutaneously into the mice. After 2 weeks, the Matrigel plugs were harvested from the mice and further analysis was performed. By immunofluorescence staining of the Matrigel plug sections with endothelial marker PECAM-1, we showed that SMC-CD34-ECs displayed the capacity to form capillary-like structures in transplanted Matrigel plugs (Figure **3.32**).

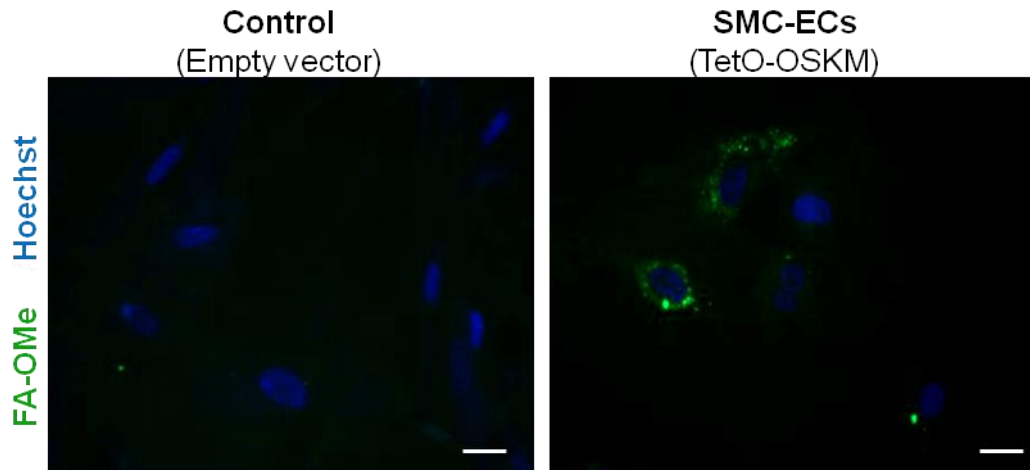


Figure 3.29 Cellular NO production assay on SMC-ECs

By using a specific probe FA-OMe to sense cellular NO, SMC-ECs started to produce NO compared to empty vector control cells. Scale bar: 35 μ m. SMCs transfected with empty lentiviral vector that underwent the same reprogramming and differentiation protocol were used as the control group.

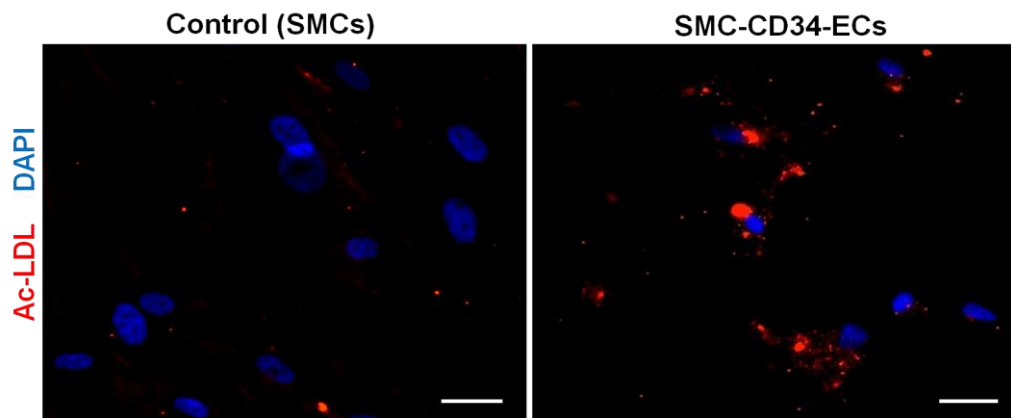


Figure 3.30 Ac-LDL uptake assay of SMC-CD34-ECs

SMC-CD34-ECs were able to efficiently take up fluorescently labelled ac-LDL compared to the control SMCs. Scale bar: 25 μ m

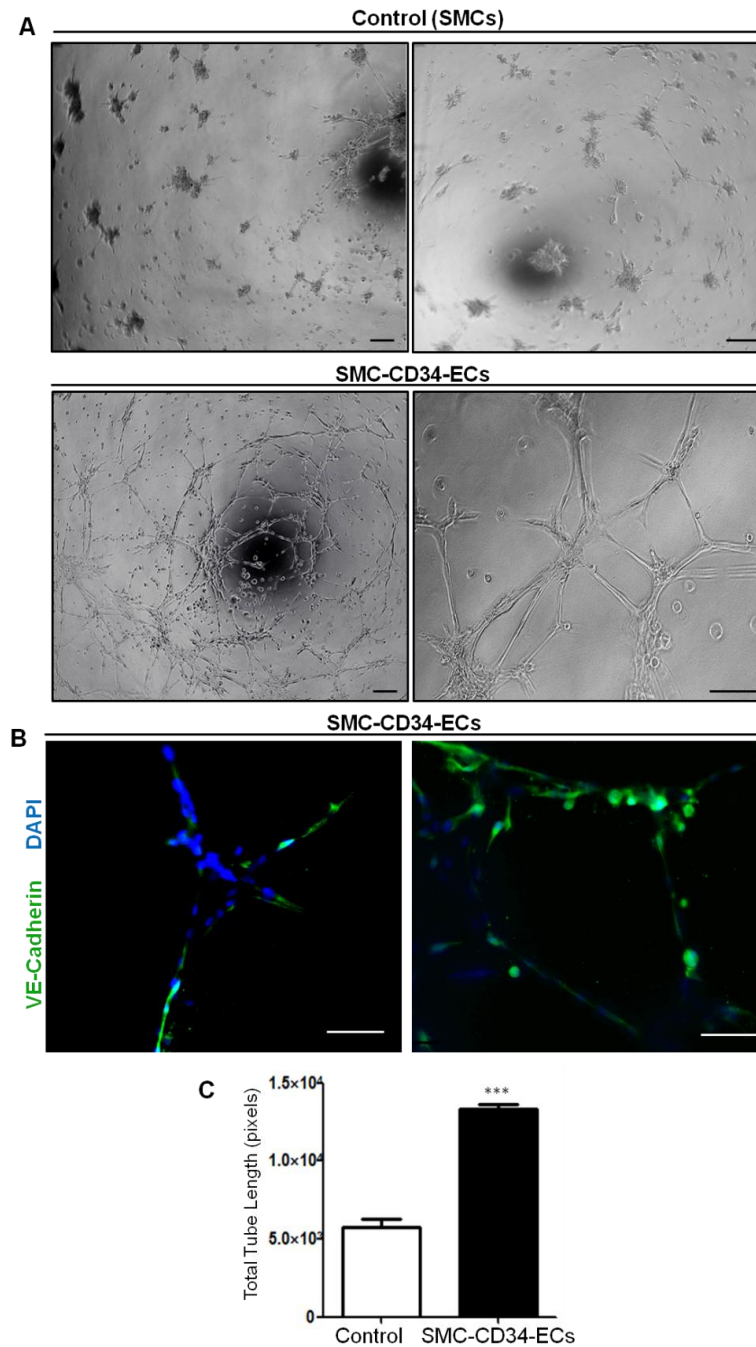


Figure 3.31 *In vitro* Matrigel angiogenesis assay of SMC-CD34-ECs

(A) *In vitro* tube formation assay was performed on control SMCs and SMC-CD34-ECs. SMC-CD34-ECs showed a much higher efficiency for tube structures forming compared to control SMCs after 6 hours seeded on Matrigel. Scale bar: 100 μ m. **(B)** The tube forming cells of SMC-CD34-ECs group are immuno-stained positive for endothelial marker VE-Cadherin. Scale bar: 100 μ m. **(C)** Total tube length quantified with Image J software displayed a significant increase with SMC-CD34-ECs group compared to control SMCs. (***) $p < 0.001$ by Student's *t* test, $n=5$)

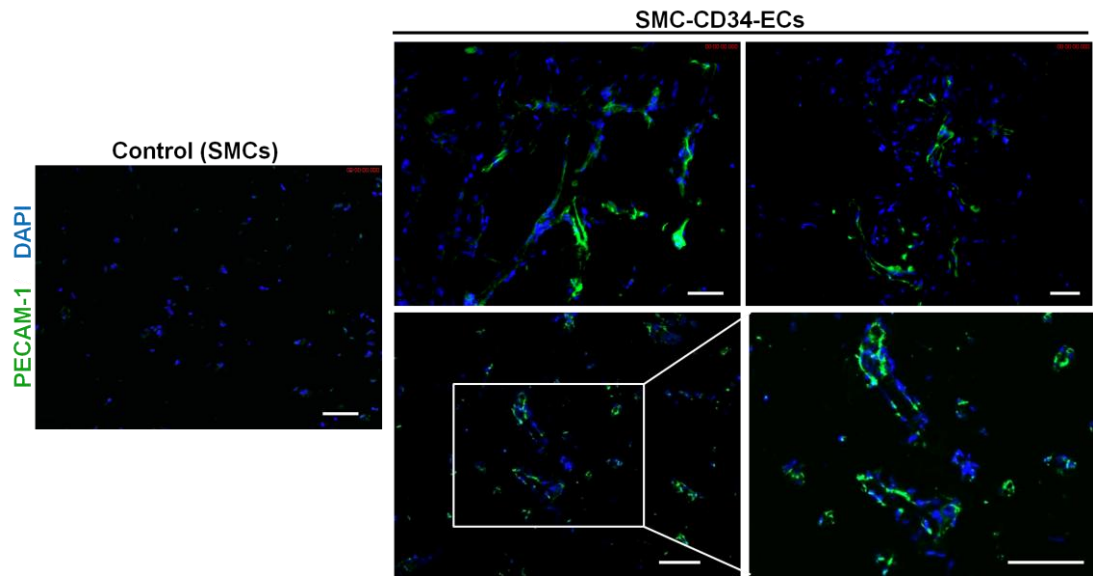


Figure 3.32 *In vivo* Matrigel angiogenesis assay of SMC-CD34-ECs

5×10^5 SMC-CD34-ECs or SMCs were mixed well with media and Matrigel and injected subcutaneously into the mice. After 2 weeks, the Matrigel plugs were harvested from the mice and immunofluorescence staining with PECAM-1 was performed on the plug sections. SMC-CD34-ECs displayed the ability to form vascular-like tubes within the plugs. Scale bar: 100 μ m.

3.2.2 SMC-derived ECs Display Therapeutic Angiogenic Capacity in a Murine Hindlimb Ischaemia Model

The murine hindlimb ischaemia model is a commonly used animal model to evaluate pro-angiogenic therapies for ischaemic diseases. The femoral artery of the mouse is ligated to create ischaemia of the lower extremity. By injecting cells or drugs locally into the ischaemic muscle, the therapeutic angiogenic capacity of treatments can be assessed straightforwardly by monitoring of blood flow recovery.

Adopting this model, the therapeutic angiogenesis potential of SMC-derived ECs was tested. Trials of three treatment groups were conducted: a SMC-CD34-ECs treated group, a SMCs treated group and a PBS treated group. SMC-CD34-ECs, SMCs or PBS were injected intramuscularly into the ischaemic hindlimbs of mice right after the ischaemic area had been created. The blood flow ratio was evaluated immediately after the surgeries by laser Doppler imaging. After two weeks, the blood perfusion recoveries of the ischaemic hindlimbs from different treatment groups were evaluated again. The therapeutic value of SMC-CD34-ECs treatment was assessed by comparison of the blood flow recoveries with PBS or SMCs injected groups. SMC-CD34-ECs treated mice showed a significantly improved blood flow ratio compared to the PBS or SMCs treated groups (Figure 3.33).

Hindlimb ischaemic area muscles were then harvested for cryosectioning and further analysis. To evaluate the total vascular structure difference, local adductor muscular tissue harvested from different treatment group and stained with mouse PECAM-1 (Margariti, Winkler et al. 2012). Images showed a clearer vascular structure in SMC-CD34-ECs treated group and quantification analysis confirmed a significant enhancement of capillary numbers of the SMC-CD34-ECs treated group compared to SMCs or PBS treated group (Figure 3.34 A, B). Furthermore, positive staining of human-specific PECAM-1 antibody within the capillaries suggested the successful engraftment of the injected human cells into *in situ* neovascularisation (Figure 3.34 C). All together, SMC-converted ECs exhibited angiogenic capacity in the mouse ischaemic model, which indicated a promising therapeutic alternative cell source for treating ischaemia diseases.

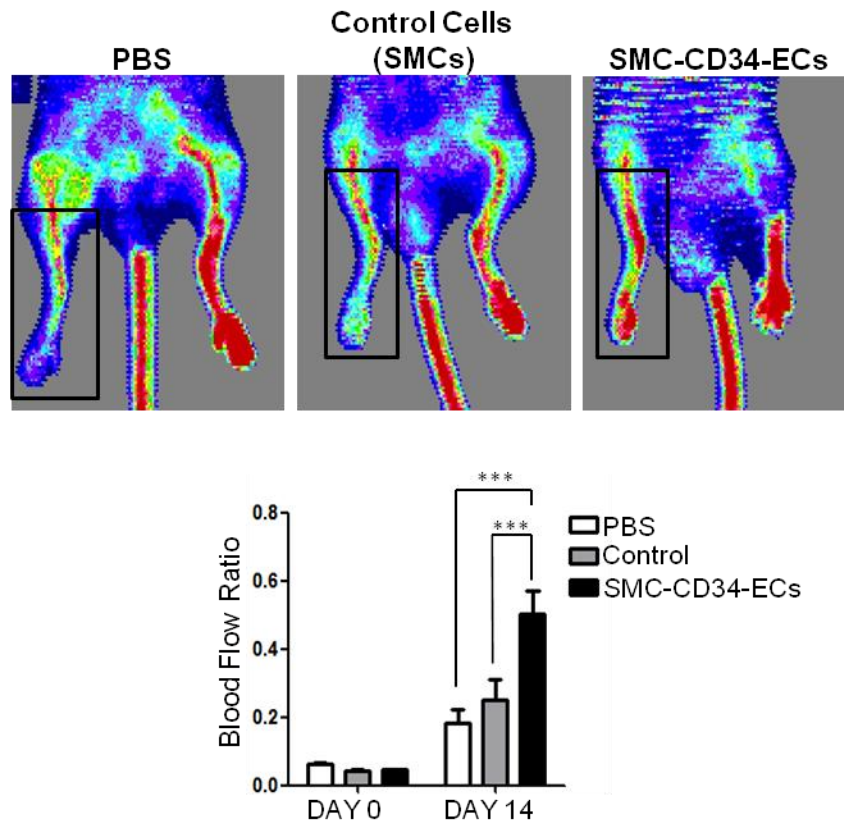


Figure 3.33 Blood flow ratio recovery of ischaemic hindlimb after two weeks of treatment

PBS, SMCs and SMC-CD34-ECs were intramuscularly injected into ischaemic hindlimbs of mice. Representative images from laser Doppler imaging exhibited that SMC-CD34-ECs injected group had a much better blood flow recovery after two weeks compared to PBS or SMCs injected group. The colour scale from blue to red indicates the increase in blood flow. Quantification analysis of blood flow ratio showed significantly higher foot blood flow recovery with SMC-CD34-ECs treated group (** $p < 0.001$ by one-way ANOVA followed by multiple comparisons with Bonferroni's method, $n=6$).

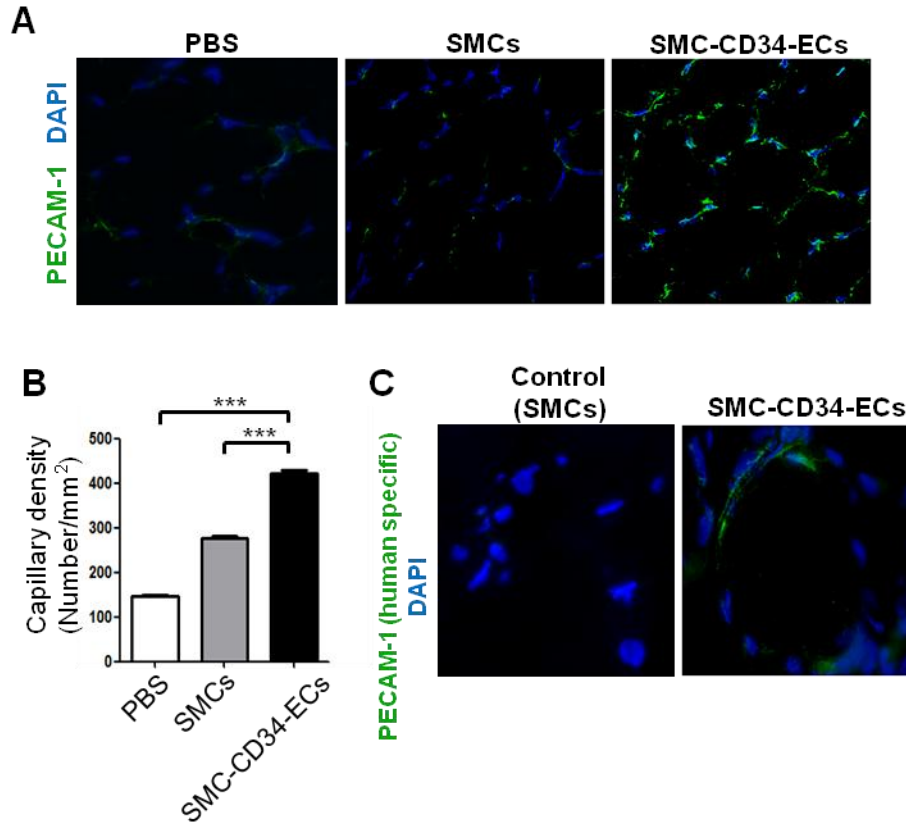


Figure 3.34 Analyses of the capillary density of the ischaemic area adductors tissue

(A) Sections of ischaemic area adductor muscles were immunofluorescence stained with mouse PECAM-1 antibody to show the total capillary structures. The SMC-CD34-ECs injected group showed significantly clearer capillary structures when compared to the control SMCs and PBS injected group. **(B)** The capillary density of the SMC-CD34-ECs treated group is much higher than PBS or SMCs treated groups. Capillary density was quantified as the capillary number per mm² (**p<0.001 by one-way ANOVA followed by multiple comparisons with Bonferroni's method, n=3) **(C)** The incorporation of SMC-CD34-ECs, which are of human origin, with *in situ* capillary formation was confirmed by the human-specific PECAM-1 immunofluorescence staining.

3.2.3 SMC-derived ECs Demonstrate the Capacity to Construct Tissue Engineered Vascular Graft

Tissue engineered vascular grafts represent a valuable prospect for regenerative medicine. It is of great significance to explore suitable materials and techniques to generate functional vascular conduits. An *ex vivo* circulation bioreactor system developed in our lab has been used to seed cells to the decellularised mouse aortic graft to construct native vessel-comparable vascular graft (Wong, Hong et al. 2015). Vascular grafts generated using this method have been transplanted *in vivo* and demonstrate normal vascular function (Karamariti, Margariti et al. 2013).

Mouse aorta was first treated with chemical and mechanical methods to remove all the cells within the vascular wall. SMC-CD34-ECs were then seeded inside the decellularised vascular graft to form the endothelial layer and SMCs were seeded on the outside. Following culturing in the bioreactor circulation system for 5 days, the graft was harvested, sectioned and immunofluorescence stained for endothelial marker PECAM-1 and SMC marker α -SMA. The reconstructed vascular graft displayed a vascular like structure with most inner endothelium-like layer formed by SMC-CD34-ECs that are surrounded by multiple SMC layers (Figure 3.35).

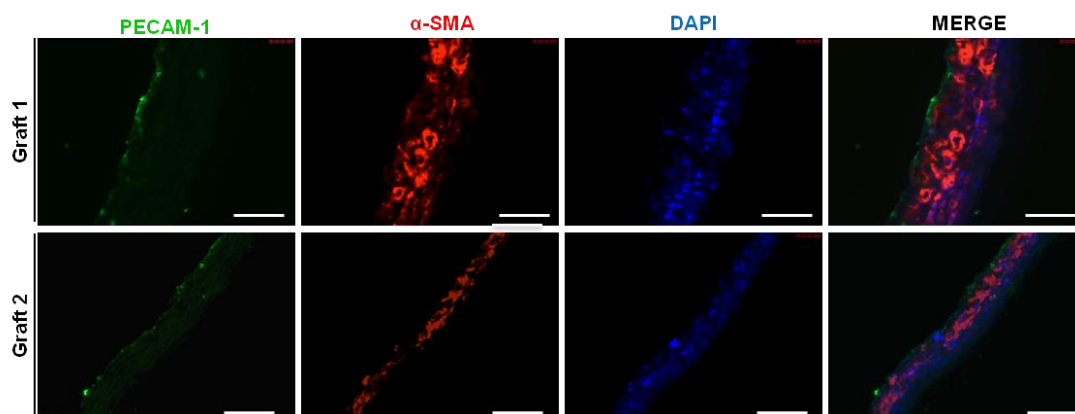


Figure 3.35 Application of SMC-CD34-ECs in constructing tissue engineered vascular grafts

Decellularised mouse aortic graft was double seeded with SMC-CD34-ECs and SMCs. After 5 days of maintenance in the *ex vivo* bioreactor setting, the graft exhibited a vascular-like structure with the inner endothelium-like layer and multiple smooth muscle layers. Two re-constructed vascular grafts were shown here from two independent experiments. Scale bar: 50 μm .

3.2.4 Conclusion

In this part of the work, the EC-related function of the SMC-derived EC has been tested. Furthermore, the application potential of the converted ECs has been evaluated for therapeutic angiogenesis in a mouse hindlimb ischaemia model and in the *ex vivo* circulation bioreactor system for the ability to construct tissue-engineered vascular graft.

SMC-derived ECs exhibit the ability to produce vasodilator NO and to take up acetylated-LDL. They also show vasculogenic and angiogenic potential *in vitro* and *in vivo*. By using the murine hindlimb ischaemia model, we demonstrated the therapeutic angiogenic capacity of SMC-CD34-ECs to help with recovering the blood flow ratio of an ischaemic area. In addition, SMC-CD34-ECs were able to construct an endothelium-like layer in a tissue-engineered vascular graft.

These functional analyses show that the ECs derived from SMCs not only express the typical endothelial markers, but also obtain endothelial-related functions and exhibit potential for further clinical application.

3.3 Mesenchymal-to-Epithelial Transition (MET) is Involved in Short Term Reprogramming to Induce a Vascular Progenitor State from SMC

3.3.1 MET Occurs Simultaneously with the Induction of Vascular Progenitors Markers in SMCs

After functionally characterising the SMC-derived endothelial population, we started to explore possible mechanisms underlying the conversion. It has been well established that mesenchymal-to-epithelial transition (MET) is an important event for the modulation of cellular plasticity. MET is essential in the initial stage of the successful reprogramming of somatic cells to iPS cells (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010). SMCs can be considered as mesenchymal-like cell due to their lack of intercellular adhesion, junction contacts and their ability to migrate through ECM. The four iPS-generating reprogramming factors were used in this study to induce SMCs to convert into the CD34-positive vascular progenitors. Morphologically, SMCs lost their mesenchymal-like shape during reprogramming (Figure 3.5). For these reasons, we checked the possible involvement of MET in the short term reprogramming phase.

When comparing vascular progenitor state PR-SMCs to SMCs, RNA-Seq data revealed an overall MET tendency, as the decrease in levels of the typical mesenchymal markers corresponded with an increase of the epithelial markers at the transcriptome level (Figure 3.36). Gene ontology analysis of the upregulated gene profile of PR-SMCs revealed an enrichment in structural molecule activity (Figure 3.37), which echoes a recent report that cytoskeletal remodelling is a key process for MET and reprogramming (Sakurai, Talukdar et al. 2014).

Based on the above results, the participation of MET during the induction of the vascular progenitor state was further investigated. The changes in mesenchymal or epithelial associated markers were measured at the mRNA level during the reprogramming from SMCs to PR-SMCs. Mesenchymal related genes α -SMA, Fibronectin and SNAIL were time-dependently downregulated accompanied with the

gradual upregulation of epithelial related genes E-Cadherin, Claudin 1 and Mucin 1 (Figure 3.38). Western blot analysis confirmed the induction of E-Cadherin and the reduction of N-Cadherin and SNAI1 at the protein level during reprogramming (Figure 3.39). PR-SMCs stained positively for E-Cadherin further demonstrating the acquisition of epithelial characteristics in parallel with reprogramming (Figure 3.40). All these results suggest that MET occurs simultaneously with the induction of vascular progenitors from SMCs.

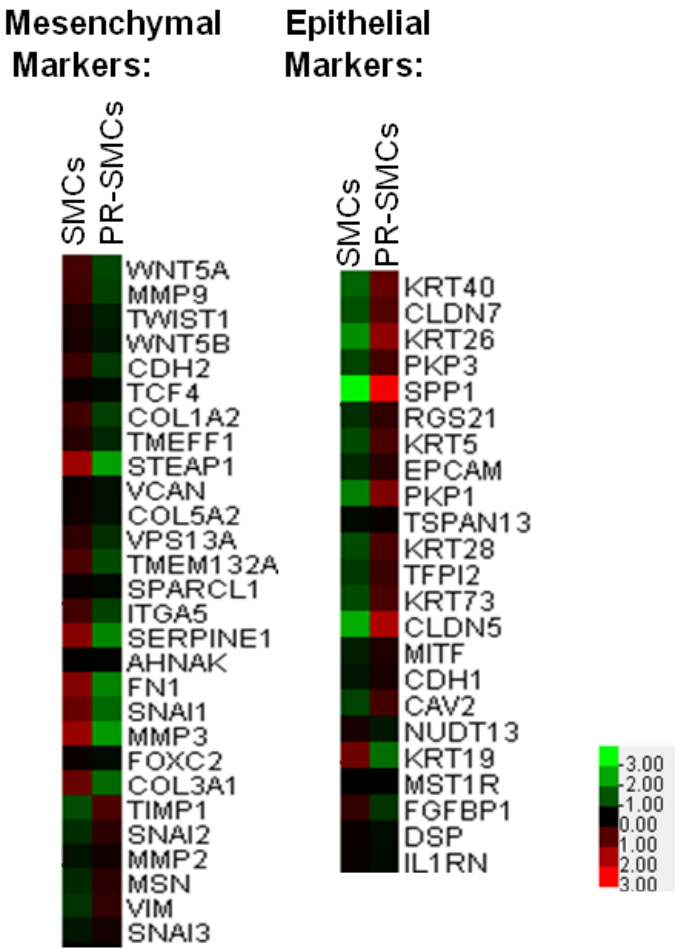


Figure 3.36 RNA-Seq results indicated a MET tendency during 4 day reprogramming

The heat map based on RNA-Seq data shows the overall suppression of mesenchymal gene profile and activation of epithelial gene profile of PR-SMCs compared with SMCs. Colour bar indicates gene expression in scale.

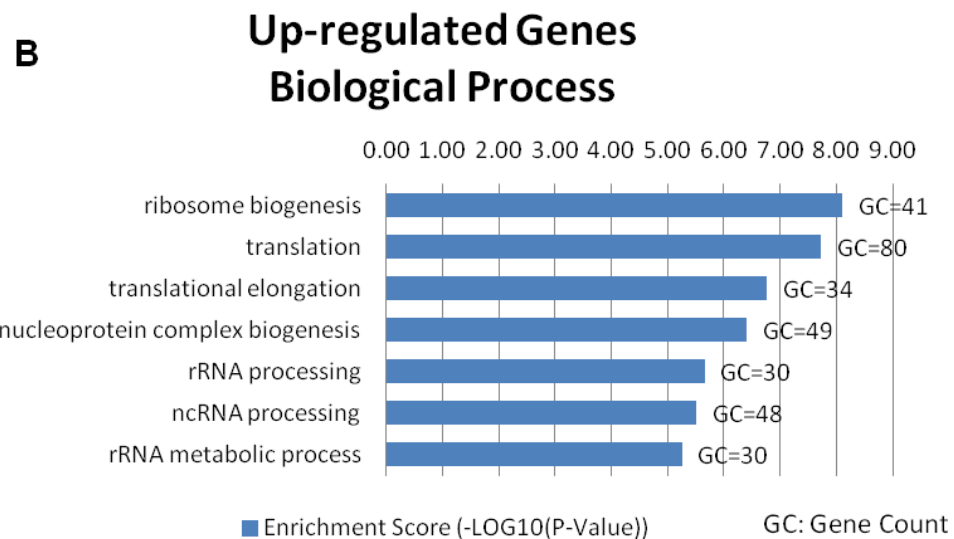
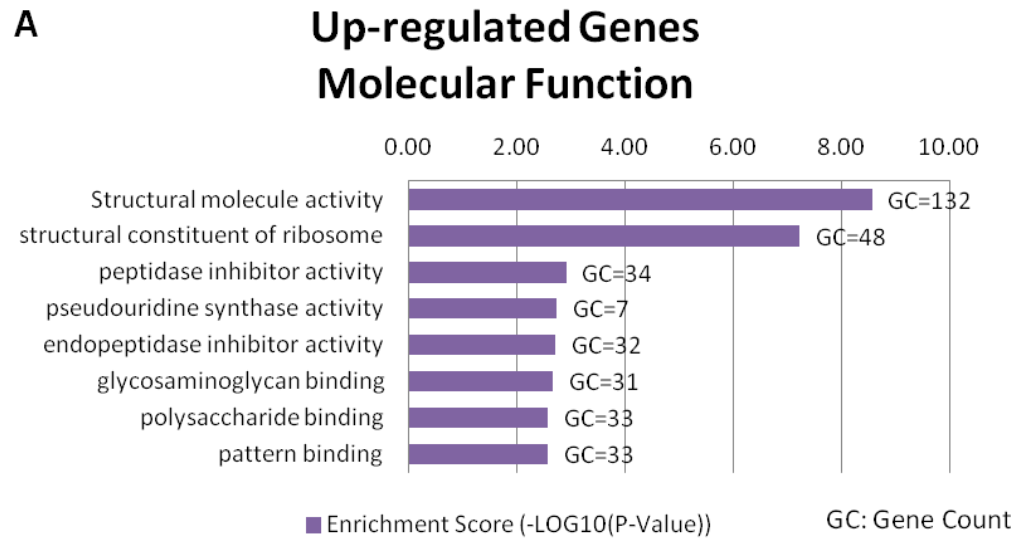


Figure 3.37 Gene ontology analysis of the upregulated genes between PR-SMCs and SMCs

Gene ontology analysis was performed on the upregulated genes of PR-SMCs compared to SMCs from the RNA-Seq data. An online DAVID bioinformatics resource 6.7 functional annotation tool suite was used to carry out the GO analysis. Enrichment in gene ontology of **(A)** biological process and **(B)** molecular function were revealed. Significance of enrichment was evaluated by p-value which is calculated by the software. The closer of p-value to zero, the more significant of the GO term annotated to the genes.

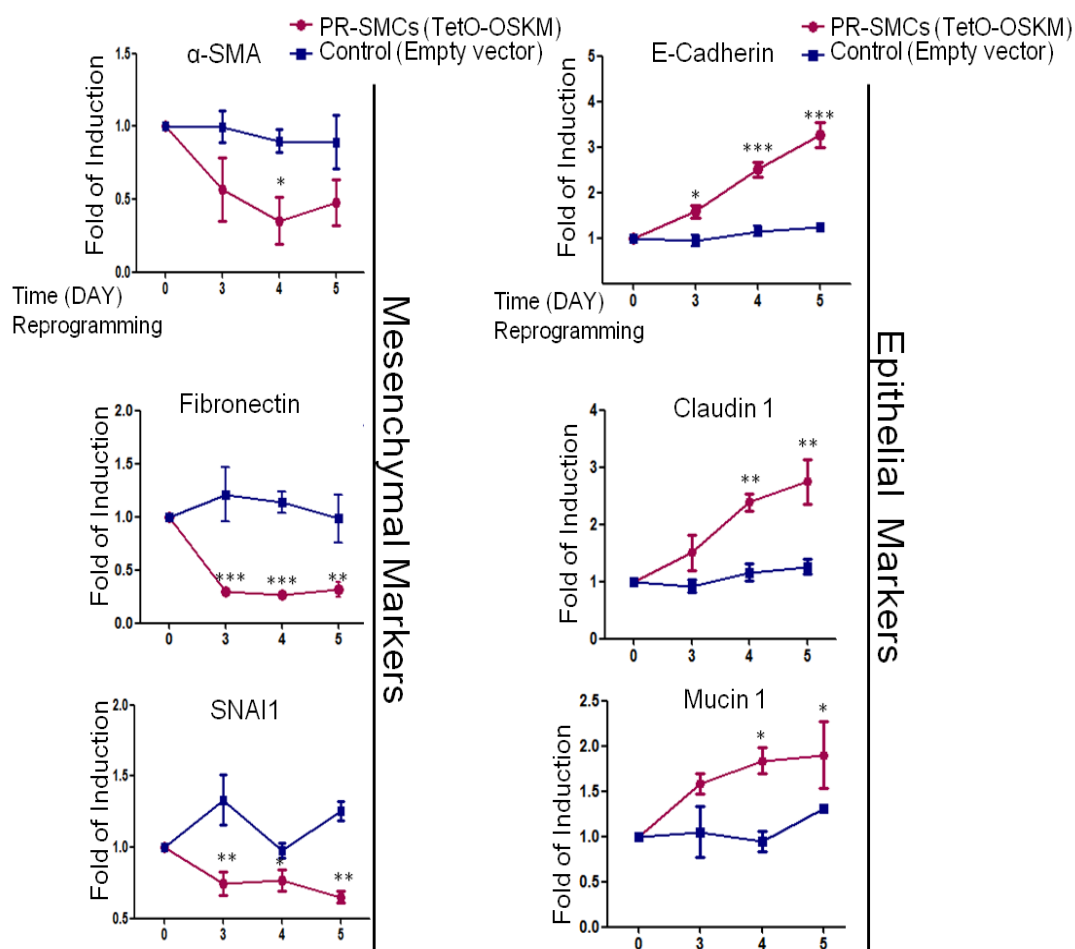


Figure 3.38 Real-time PCR analyses of representative mesenchymal and epithelial markers during reprogramming

Real-time PCR results showed that during SMCs to PR-SMCs reprogramming, mesenchymal markers SMA, Fibronectin, and SNAI1 were gradually downregulated and epithelial markers E-Cadherin, Claudin 1, and Mucin 1 were gradually induced (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA followed by multiple comparisons with Bonferroni's method, $n = 3$). SMCs transfected with empty lentiviral vector without the four reprogramming factors that underwent the same reprogramming process were treated as the control group.

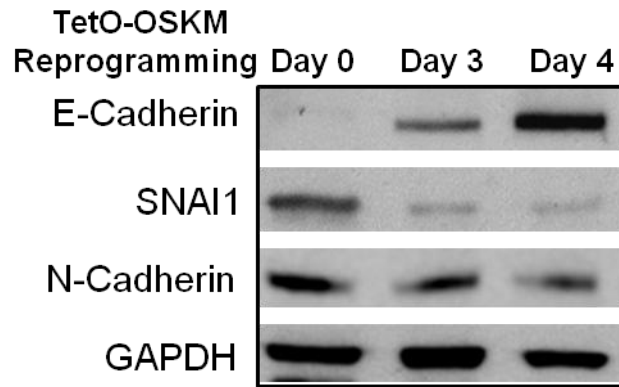


Figure 3.39 Western blot analysis of the expression changes of typical epithelial and mesenchymal markers during reprogramming

After the overexpression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC*, the upregulation of epithelial marker E-Cadherin and suppression of mesenchymal markers SNAI1 and N-Cadherin during reprogramming were confirmed at the protein level by western blot analysis.

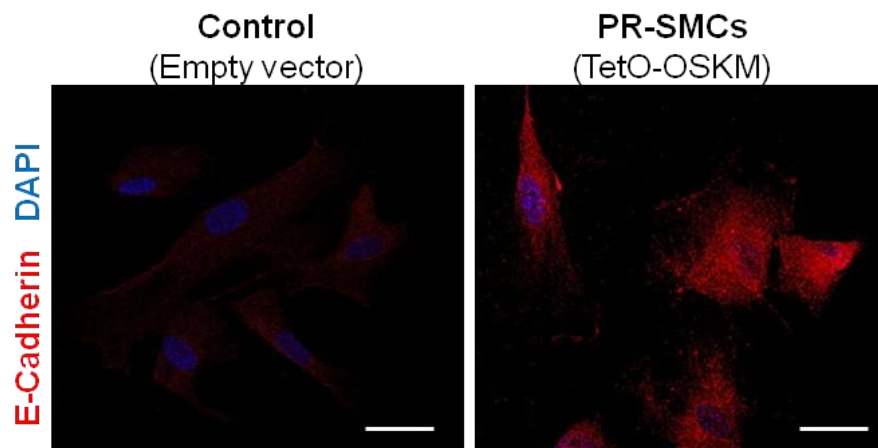


Figure 3.40 Immunofluorescence staining of PR-SMCs for typical epithelial marker E-Cadherin

PR-SMCs and empty vector control cells were immunofluorescence stained with E-Cadherin. The expression of E-Cadherin could be observed in PR-SMCs (Scale bar: 25 μ m). SMCs transfected with empty lentiviral vector without the expression of the four factors that underwent the same reprogramming protocol were used as the control group.

3.3.2 Knockdown of E-Cadherin Impairs the Generation of Vascular Progenitors from SMCs and the Further Endothelial Lineage Differentiation

E-Cadherin is the key regulator for epithelial homeostasis and knocking down E-Cadherin at the initial stage of iPS cell generation impairs iPS cell induction (Li, Liang et al. 2010). To determine whether a shift of the cell state towards epithelial-like cell is indispensable in the generation of PR-SMCs and subsequent endothelial lineage induction, we intended to knockdown E-Cadherin during reprogramming. The knockdown of E-Cadherin was achieved via lentiviral delivery of short hairpin E-Cadherin (shECAD) during reprogramming. Cells transfected with Non-Target shRNA (shNT) were used as the control to eliminate off-target effects. SMCs transfected with the four reprogramming factors were kept under reprogramming conditions for 2 days before transfection with shECAD or shNT. Cells were then maintained under the same reprogramming conditions for a further 2 days after transfection. Real-time PCR analysis showed that the CD34 expression was repressed corresponded with the suppression of E-Cadherin expression in PR-SMCs (Figure 3.41 A), which indicated that the generation of a vascular progenitor state was compromised due to the interruption of the induction of epithelial properties.

To evaluate the impact of E-Cadherin knockdown during reprogramming on subsequent endothelial differentiation, PR-SMCs with or without E-Cadherin knockdown were subjected to the endothelial-promoting condition for 6 days. Real-time PCR indicated that the successive endothelial differentiation capacity of PR-SMCs was jeopardised with E-Cadherin knockdown as the upregulation of endothelial markers was impaired (Figure 3.41 B).

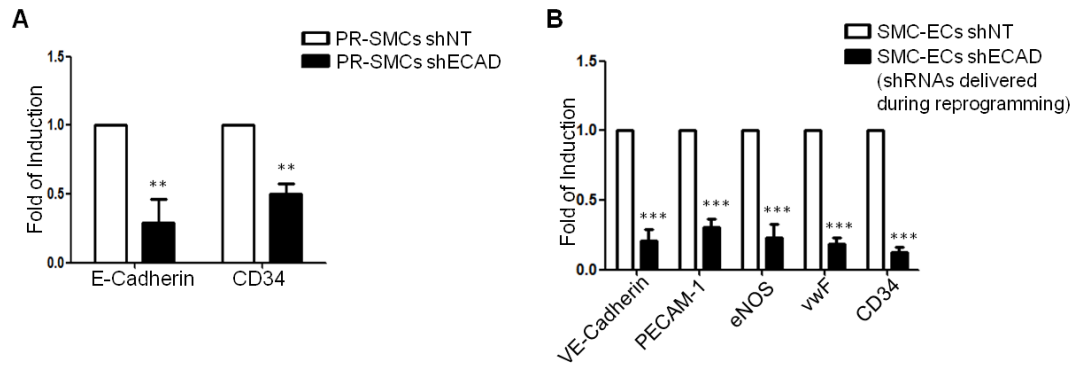


Figure 3.41 Knockdown of E-Cadherin during reprogramming impaired the generation of vascular progenitors and further induction of endothelial properties

(A) Real-time PCR results indicated that knockdown of E-Cadherin with shRNA at day 2 of reprogramming resulted in the downregulation of CD34 in PR-SMCs at the mRNA level (** $p < 0.01$ by Student's t test, $n=3$). **(B)** When PR-SMCs with or without E-Cadherin knockdown were subjected to endothelial differentiation, PR-SMCs with E-Cadherin knockdown displayed impaired capacity for endothelial marker upregulation (** $p < 0.001$ by Student's t test, $n=3$).

3.3.3 Conclusion

In this section of the work, the mechanism involved in the conversion of SMCs to vascular progenitor state PR-SMCs was explored. During the reprogramming from SMCs towards PR-SMCs, MET was identified as a key cellular process. The downregulation of mesenchymal properties and the induction of epithelial properties were observed along the reprogramming process. Furthermore, when we knocked down the crucial epithelial regulator E-Cadherin during reprogramming, the generation of CD34-positive vascular progenitor PR-SMCs was impaired as was the further differentiation towards an endothelial lineage.

Taken together, during 4 days reprogramming phase, MET along with the induction of E-Cadherin are required events for the generation of vascular progenitor from SMCs and necessary for the further differentiation towards an endothelial lineage.

3.4 Components of the Notch Signalling Pathway, HES5 and JAG1, are Implicated in Endothelial Lineage Derivation from PR-SMC

3.4.1 Comprehensive Analyses Reveal the Involvement of HES5 and JAG1 during PR-SMC to Endothelial Lineage Differentiation

The Notch signalling pathway plays an important yet complex role in regulating differentiation, proliferation, angiogenesis and other cellular processes of both EC and SMC (Gridley 2007). RNA-Seq analysis indicated that upregulation of several components of the Notch pathway occurred with the generation of SMC-derived ECs (Figure 3.42), which led to our particular interest in exploring the participation of the Notch pathway in endothelial differentiation. To verify the results from RNA-Seq, Real-time PCR analysis was performed to check the gene expression levels of the Notch pathway components that were shown to be upregulated in SMC-derived ECs from RNA-Seq data or have been reported to be associated with EC (Figure 3.43). Collectively, the results showed a consistent upregulation of HES5 and JAG1 during vascular progenitor to endothelial lineage differentiation. Furthermore, western blot analysis confirmed the upregulation of JAG1 and HES5 aligned with the increase of endothelial marker at the protein level (Figure 3.44). Taking all these into account, we decided to look at the possible regulatory roles of HES5 and JAG1 during endothelial differentiation.

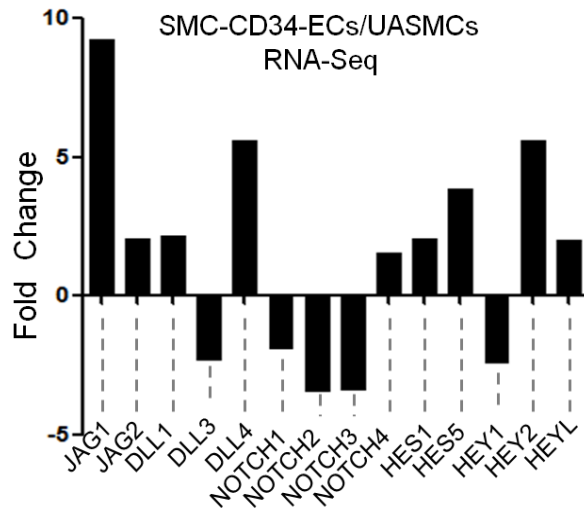


Figure 3.42 Gene expression fold changes of the Notch signalling pathway members

RNA-Seq analysis showed the gene expression fold changes of the Notch signalling pathway members in SMC-CD34-ECs compared to SMCs. Fold changes were calculated based on the normalised RPKM value of each gene in different cell samples.

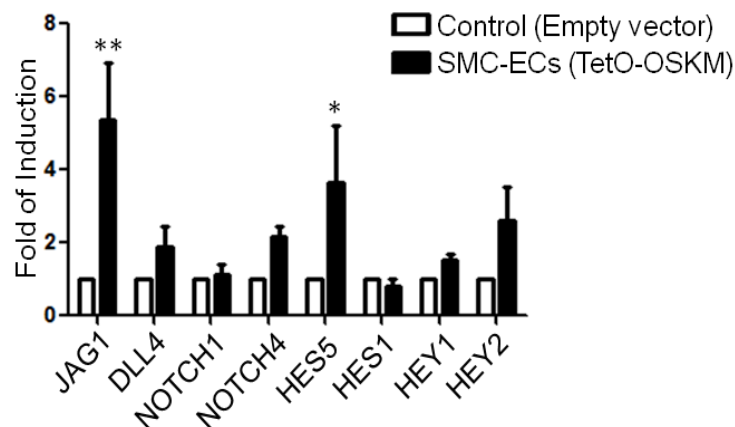


Figure 3.43 Real-time PCR analysis of the Notch pathway components in SMC-ECs

Real-time PCR was performed to verify the upregulation of the Notch signalling pathway members from the RNA-Seq results plus other Notch pathway components that have been reported to be associated with EC. JAG1 and HES5 were upregulated in SMC-derived ECs (* $p < 0.05$, ** $p < 0.01$)

by Student's *t* test, *n*=3). SMCs transfected with empty lentiviral vector without 4 factors expression that underwent the same reprogramming and differentiation protocol were used as the control group.

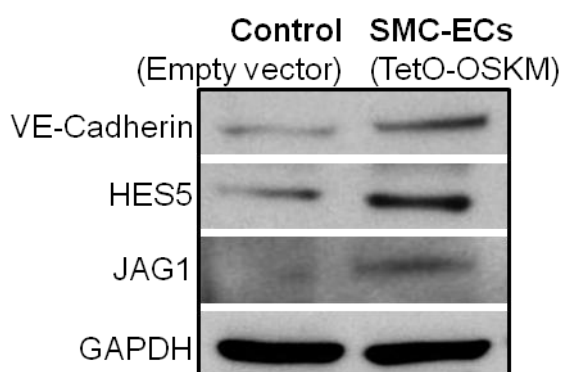


Figure 3.44 Western blot analysis of HES5 and JAG1 expression in SMC-ECs

Western blot analysis showed the upregulation of HES5 and JAG1 along with the induction of endothelial markers in SMC-ECs. SMCs transfected with empty lentiviral vector without the 4 factors expression that underwent the same reprogramming and differentiation protocol were used as the control group.

3.4.2 HES5 is Required for the Endothelial Lineage induction from PR-SMC

HES5 is a downstream target of the Notch signalling pathway and functions as a transcription factor of the basic helix-loop-helix family (Kageyama, Ohtsuka et al. 2007). HES5 has been associated with regulating the maintenance and the differentiation of progenitor cells of various organs and tissues. Several recent studies indicated a role for HES5 in cardiovascular development. Xu *et al.* suggested that HES5 might act as a key positive mediator for the differentiation of bone marrow stromal cell into EC (Xu, Liu et al. 2009). HES5 has also been implicated in endothelial proliferation in response to endothelial injury during atherosclerosis (Schober, Nazari-Jahantigh et al. 2014).

To elucidate the involvement of HES5 during endothelial generation from PR-SMC, loss-of-function and gain-of-function analyses of HES5 were performed. PR-SMCs were treated with endothelial-promoting conditions to differentiate them towards an endothelial lineage for 3 days before transfection with lentivirus short hairpin HES5 (shHES5) to suppress HES5 expression. Cells transfected with Non-Target shRNA (shNT) were used as a control. ShHES5 or shNT transfected cells were maintained in the same endothelial-promoting conditions for another 3 days. ShHES5-mediated HES5 knockdown resulted in the reduction of endothelial markers at the mRNA and the protein levels (Figure 3.45), which demonstrated that a lack of HES5 impaired the regulation of the key processes for endothelial differentiation.

Next, HES5 was overexpressed at day 3 of PR-SMCs to endothelial differentiation via delivering HES5-encoding plasmid, pCMV6-HES5, into the cells. Cells transfected with empty pCMV6 plasmid were used as the control. After a further 3 days of endothelial differentiation, HES5 overexpressing SMC-ECs exhibited a further enhancement of endothelial marker expression compared to empty vector delivered SMC-ECs (Figure 3.46). Interestingly, immunofluorescence staining suggested that the positive endothelial regulatory role of HES5 only functioned during PR-SMCs to endothelial differentiation. Overexpressing HES5 in cells without prior *OCT4*, *SOX2*, *KLF4*, *c-MYC* reprogramming failed to induce

endothelial marker expression (Figure **3.47**). The above findings imply that the four reprogramming factors introduced certain frames for HES5's further regulation of expression of endothelial related genes.

We next wanted to check the possible targets of HES5 during endothelial differentiation. eNOS was the most highly induced endothelial marker during PR-SMCs to SMC-ECs differentiation and eNOS could be induced to a higher level upon HES5 overexpression. Therefore, a luciferase assay was performed to check the potential regulatory role of HES5 on the promoter region of eNOS. PR-SMCs were first treated with endothelial-promoting condition for 3 days, and then transfected with the plasmid expressing luciferase under the control of eNOS promoter with the HES5-overexpressing plasmid or empty control plasmid. Luciferase activity was detected 48 hours after transfection. Result showed that the activity of the eNOS promoter was enhanced in the HES5-overexpressing cells (Figure **3.48**). Thus, HES5 benefits the endothelial differentiation at least partly through activation of the eNOS promoter. HES5 might directly bind to the eNOS promoter region to activate it or act on the promoter through other factors.

In addition, the expression of Hes5 was assessed in a mouse iPS cell to EC differentiation model. Mouse iPS cells were seeded on Collagen IV coated flasks and maintained in differentiation media supplemented with 50 ng/ml VEGF to induce endothelial differentiation. Hes5 expression was upregulated along with the induction of endothelial markers at the mRNA and the protein level (Figure **3.49**). This interesting finding further indicated that HES5 might play an extended regulatory role in endothelial differentiation in general.

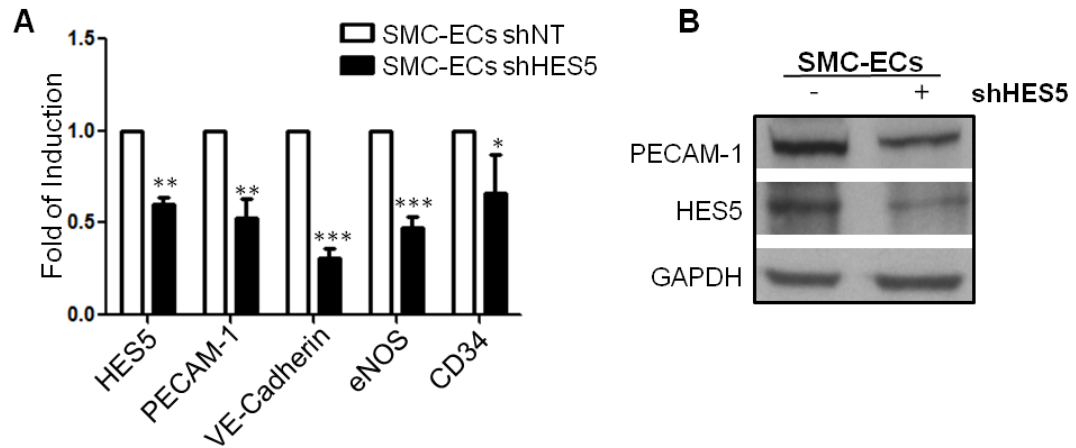


Figure 3.45 HES5 knockdown impaired the induction of endothelial markers in SMC-ECs

(A) Real-time PCR results showed the knockdown of HES5 led to the impairment of endothelial marker induction in SMC-ECs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's *t* test, $n = 3$) **(B)** The suppression of endothelial marker PECAM-1 with HES5 knockdown in SMC-ECs was confirmed at the protein level by western blot analysis. SMCs transfected with empty lentiviral vector without the 4 factors expression that underwent the same reprogramming and differentiation protocol were used as the control group for SMC-ECs.

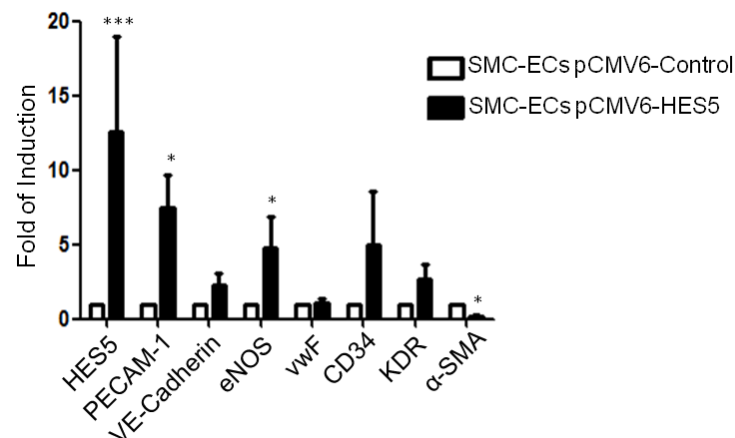


Figure 3.46 Overexpression of HES5 in SMC-ECs further enhanced the expression of endothelial markers

Overexpression of HES5 in SMC-ECs through plasmid delivery of pCMV6-HES5 led to a further increase of the expression of endothelial markers at

the mRNA level compared to empty pCMV6 vector transfected SMC-ECs (* $p < 0.05$, *** $p < 0.001$ by Student's t test, $n=3$)

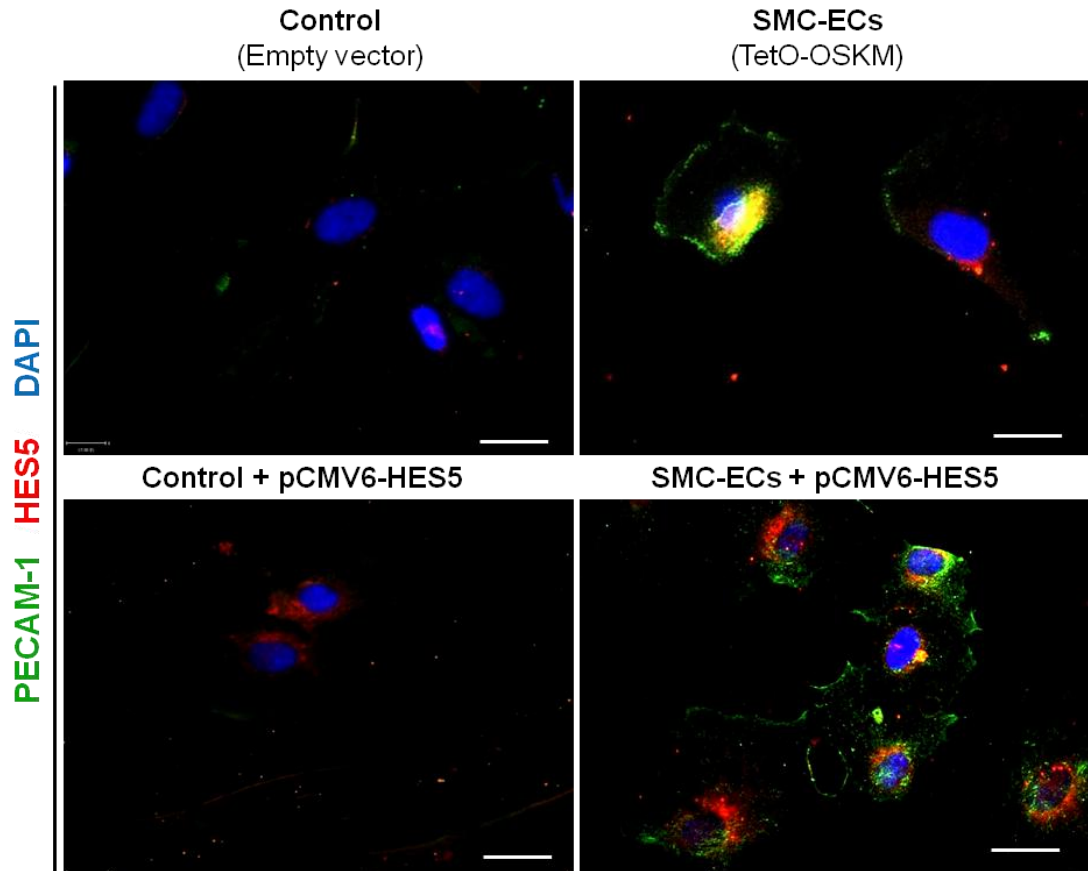


Figure 3.47 Immunofluorescence staining of the control cells or SMC-ECs with HES5 overexpression

Immunofluorescence staining of HES5 and PECAM-1 was performed on control cells (SMCs transfected with empty lentiviral vector without 4 factors expression that underwent the same reprogramming and differentiation protocol), SMC-ECs, control cells with HES5 overexpression and SMC-ECs with HES5 overexpression. Results showed that overexpression of HES5 in SMC-ECs by transfection with plasmid pCMV6-HES5 further induced the PECAM-1 expression compared to the empty pCMV6 vector transfected SMC-ECs. However, overexpression of HES5 in control cells that were not reprogrammed with *OCT4*, *SOX2*, *KLF4*, and *c-MYC* could not achieve the endothelial marker inducing effect. Scale bar: 25 μm .

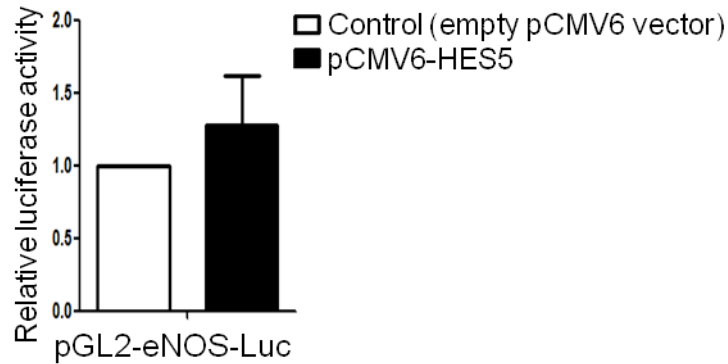


Figure 3.48 Luciferase assay of eNOS promoter activity upon HES5 overexpression in SMC-ECs

After 3 days endothelial differentiation, PR-SMCs were co-transfected with reporter plasmid pGL2 for eNOS promoter and HES5-overexpressing plasmid pCMV6-HES5 or empty plasmid pCMV6. Measurement of relative luciferase activity indicated that eNOS promoter activity was enhanced upon HES5 overexpression. Cells were transfected with a Renilla-expressing vector at the same time as the normalisation control for transfection efficiency. Relative luciferase activity is the ratio of luciferase activity to Renilla activity with empty pCMV6 vector control set as 1.0 (n=3).

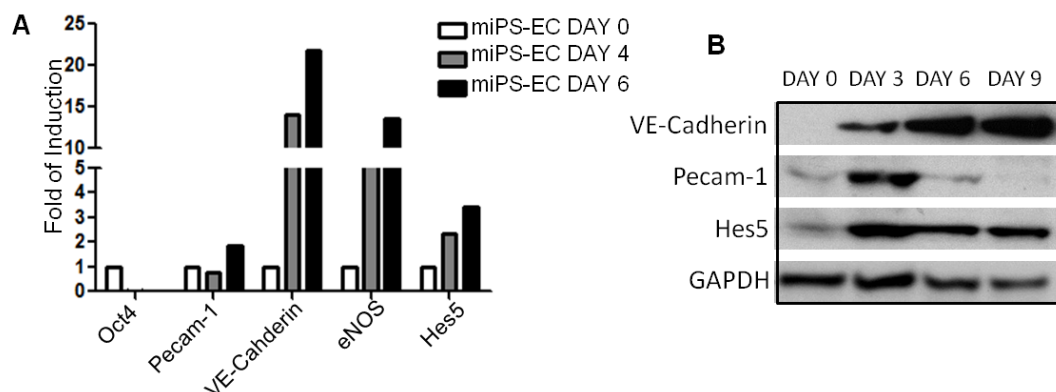


Figure 3.49 Hes5 is implicated in mouse iPS cell to EC differentiation

In a mouse iPS cell to EC differentiation model, Hes5 expression was upregulated corresponded with the increase of endothelial markers at **(A)** the mRNA level (n=2) and **(B)** the protein level.

3.4.3 JAG1 Participates in the PR-SMC to Endothelial Lineage Differentiation

JAG1 is the upstream ligand of the Notch signalling pathway which serves a much more complicated context-dependent role in regulating vascular activity (Gridley 2007, Benedito, Roca et al. 2009). A facilitating role of JAG1 in endothelial differentiation and angiogenesis has been indicated in several studies. For example, Benedito *et al.* showed the pro-angiogenic role of JAG1 during mouse embryogenesis and postnatal development through interaction with another Notch ligand Delta-like 4 (DLL4) (Benedito, Roca et al. 2009). Specific JAG1 stimulation in the bone marrow microenvironment has been shown to be critical for endothelial progenitor cell development and neovascularisation (Kwon, Eguchi et al. 2008). Considering that JAG1 has been upregulated through the endothelial profile induction from PR-SMCs, it is of great interest to further investigate the role of JAG1 during this process.

First, we stimulated the cells with recombinant JAG1 during the PR-SMCs to endothelial differentiation. PR-SMCs were treated with endothelial-promoting condition for 3 days and then seeded on immobilised recombinant JAG1 or control IgG coated petri-dishes. The cells were maintained in endothelial-promoting condition for another 2 days. Upon recombinant JAG1 stimulation, some of the endothelial markers, especially eNOS, could be induced to a higher level compared to IgG treated cells (Figure 3.50).

Next, we studied the impact of JAG1 suppression on endothelial differentiation from PR-SMCs. PR-SMCs were differentiated towards endothelial lineage for 3 days before they were transfected with short hairpin JAG1 (shJAG1) lentivirus or control shNT lentivirus. The cells were then treated with endothelial-promoting condition for another 3 days. Suppression of JAG1 through shJAG1 delivery caused impairment of the expressions of the similar group of endothelial markers that could be upregulated with recombinant JAG1 stimulation (Figure 3.51).

Previous studies showed that JAG1 stimulation could activate eNOS in the presence of VEGF (Patenaude, Fuller et al. 2014). Luciferase assay was performed to detect

the activity of eNOS promoter upon JAG1 overexpression. We observed that the activity of the eNOS promoter was increased by JAG1 overexpression (Figure 3.52).

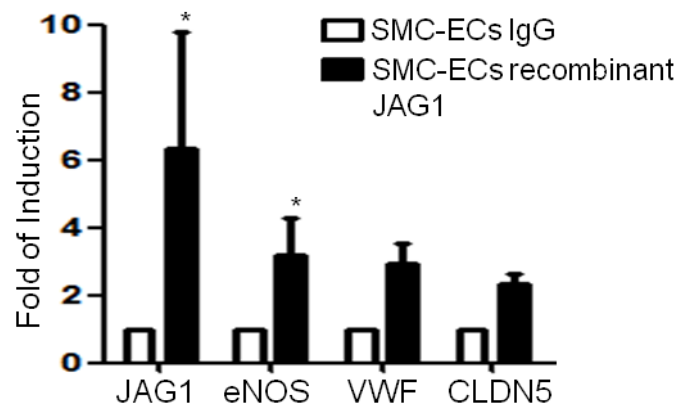


Figure 3.50 Stimulation of the cells with recombinant JAG1 led to the further upregulation of endothelial markers

Real-time PCR revealed that after stimulating the cells with immobilised recombinant JAG1 from day 3 of differentiation, endothelial markers eNOS, VWF and Claudin 5 could be further upregulated (* $p < 0.05$ by Student's *t* test, $n = 3$).

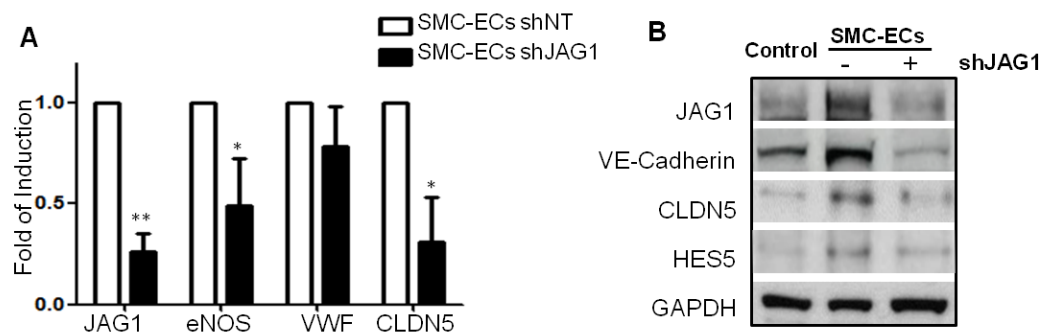


Figure 3.51 JAG1 knockdown impaired the induction of endothelial markers of SMC-ECs

(A) Real-time PCR analysis indicated that the knockdown of JAG1 led to the downregulation of levels of eNOS and Claudin 5 (* $p < 0.05$ by Student's *t* test, $n = 3$). (B) Western blot analysis confirmed the downregulation of endothelial

markers in concert with the JAG1 suppression in SMC-ECs. SMCs transfected with empty lentiviral vector without the 4 factors expression that underwent the same reprogramming and differentiation protocol were used as the control group.

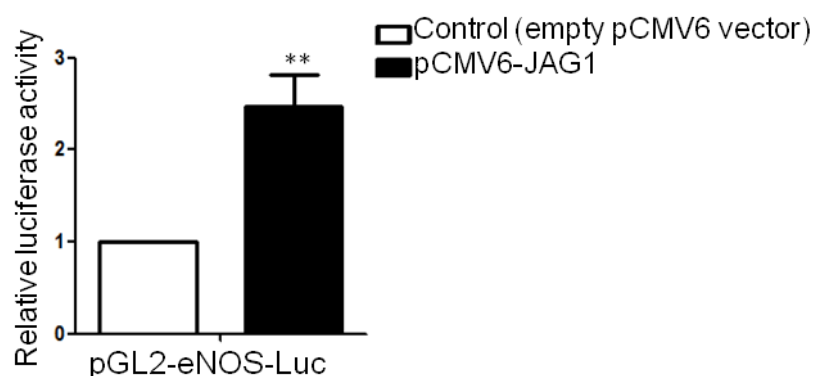


Figure 3.52 Luciferase assay of eNOS promoter activity upon JAG1 overexpression in SMC-ECs

3 day endothelial-differentiated PR-SMCs were transfected with reporter plasmid pGL2 for eNOS promoter and JAG1-overexpressing plasmid pCMV6-JAG1 or empty plasmid pCMV6. Measurement of relative luciferase activity indicated that eNOS promoter activity was enhanced upon JAG1 overexpression. Cells were transfected with Renilla-expressing vector at the same time as the normalisation control for transfection efficiency. Relative luciferase activity is the ratio of luciferase activity to Renilla activity with empty pCMV6 vector control set as 1.0 (* $p < 0.05$ by Student's t test, $n=3$).

3.4.4 JAG1 might Act through HES5 to Regulate Endothelial Differentiation from PR-SMC

HES5 has been reported to be a downstream target of JAG1 in several cellular models: hair cell and support cell differentiation (Tang, Alger et al. 2006); glioblastoma stem cells to pericyte-like cell differentiation (Guichet, Guelfi et al. 2015); lipopolysaccharide-induced inflammatory response of macrophage cells (Tsao, Wei et al. 2011) and others.

In our study, both HES5 and JAG1 participated in regulation of endothelial differentiation. Interestingly, both of them could enhance eNOS promoter activity. JAG1 knockdown led to the suppression of HES5 expression (Figure 3.51 B) during PR-SMCs to endothelial differentiation. In addition, a luciferase assay was performed to evaluate HES5 promoter activity upon the overexpression of JAG1 during endothelial differentiation. JAG1 overexpression could enhance HES5 promoter activity (Figure 3.53). The above results suggest that JAG1 might act on the downstream HES5 to carry out its endothelial regulatory function. Further work should be performed to clarify the interaction between JAG and HES5 and their detailed regulatory mechanism on eNOS promoter.

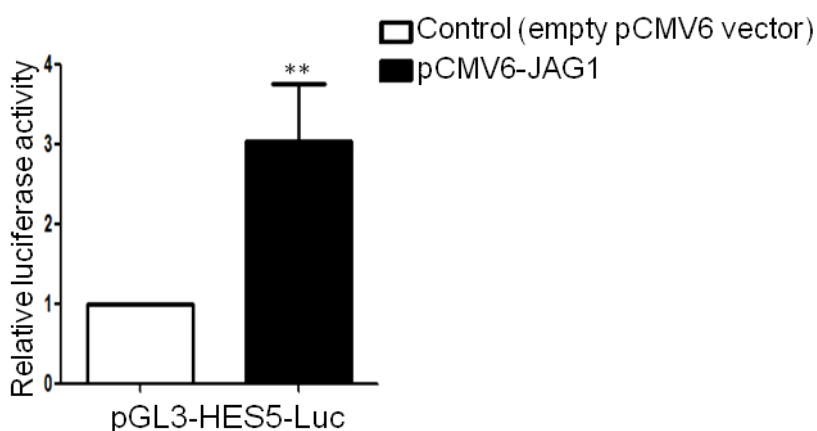


Figure 3.53 Luciferase assay of HES5 promoter activity upon JAG1 overexpression in SMC-ECs

3 day endothelial-differentiated PR-SMCs were transfected with reporter plasmid pGL3 for HES5 promoter and JAG1-overexpressing plasmid pCMV6-JAG1 or empty plasmid pCMV6. Measurement of relative luciferase activity indicated that HES5 promoter activity was enhanced upon JAG1 overexpression. Cells were transfected with Renilla-expressing vector at the same time as the normalisation control for transfection efficiency. Relative luciferase activity is the ratio of luciferase activity to Renilla activity with empty pCMV6 vector control set as 1.0 (* $p < 0.05$ by Student's t test, $n=3$).

3.4.4 Conclusion

The participation of two components of the Notch signalling pathway, HES5 and JAG1, during endothelial differentiation from PR-SMCs has been studied. Comprehensive analyses of RNA-Seq and Real-time PCR data revealed the concurrent upregulation of HES5 and JAG1 with endothelial differentiation. Knockdown or overexpression of HES5 during PR-SMCs to endothelial differentiation led to the suppression or further increase of endothelial markers respectively. HES5 regulates the activation of the eNOS promoter. Stimulating the cells under endothelial differentiation with JAG1 caused the enhancement of a group of endothelial-related genes. Knockdown of JAG1 during differentiation led to the suppression of a similar group of genes. JAG1 also played a role in modulating eNOS promoter activity. More interestingly, JAG1 could activate the promoter region of HES5.

Taken together, our study focused on revealing the contribution of HES5 and JAG1 as specific parts of the Notch signalling pathway that are involved in promoting EC differentiation.

CHAPTER 4. DISCUSSION

4.1 Brief Summary of Major Findings

In this study, we have demonstrated the feasibility of the derivation of ECs from human SMCs for the first time. The conversion was achieved using a two steps protocol consisting of first reprogramming SMCs to CD34-positive vascular progenitors and subsequently differentiating the cells towards a functional endothelial lineage. Importantly, the SMC-derived ECs display angiogenic capacity especially in an animal ischaemic disease model as well as the ability to assemble into an endothelial layer in tissue engineered vascular graft. Furthermore, our data reveal that the MET and the Notch signalling pathways are important mediators through which cell fate conversion can be attained. Our findings present the first indication that human SMCs may represent a new valuable source of functional ECs for cell-based therapy. These findings will be discussed in detail in the following paragraphs.

4.2 Establishment and Optimisation of the Protocol for the Conversion of Human SMC towards an Endothelial Fate

4.2.1 Choosing the Basic Strategy for the Conversion from SMC to EC: EC-Specific Transcription Factors or iPS cell-Generating Transcription Factors?

First, the basic strategy for the direct conversion of SMC towards an endothelial lineage needed to be decided. Currently, there are two main approaches to achieve direct cell lineage conversion. One is to use target cell type-specific transcription factors to activate the target cell type's gene regulatory network in the starting cell population. Another approach is to use iPS cell-generating reprogramming factors combined with specific culture conditions to erase the starting cells' lineage-specific signatures and to reactivate repressed transcription and epigenetic networks without the full induction of pluripotency. Cells then revert to an intermediate plastic state which permits further manipulation and new lineage commitment towards the desired cell types.

Although using the target cell-specific transcription factors for direct cell lineage reprogramming represents a straightforward strategy, a major concern related to this method is that the original gene regulatory network of the starting cell type may be insufficiently inactivated. Indeed, a recent study provided evidence to support this concern by comparing the gene expression patterns of various directly reprogrammed cell types with cells derived from pluripotent stem cells using a computational network biology platform named CellNet. Comprehensive analysis showed that directly reprogrammed cells tend to inadequately silence the expression programs of the starting cell population (Cahan, Li et al. 2014). This suggests that using target cell-specific transcriptions factors to conduct the direct cell lineage conversion may fail to fully erase the identity of the starting cell type and result in the incomplete establishment of the gene regulatory networks of the target cell type.

Instead, iPS-generating pluripotent factors can provide the cells with a clean slate for the further full induction of target cell type characteristics.

Specific to our study, SMC and EC may share common vascular progenitors under certain circumstances (reviewed in section 1.1.4.3). Therefore, it seemed more practical to use the reprogramming factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* to dedifferentiate the SMCs back to a plastic state to mimic the vascular progenitor state as a first step, and then differentiate the intermediate progenitor state cells towards an endothelial lineage.

4.2.2 Conversion of SMCs to a Plastic Intermediate State through Short Time Reprogramming

We used a lentiviral vector TetO-OSKM to efficiently overexpress *OCT4*, *SOX2*, *KLF4*, and *c-MYC* in the SMCs. Lentivirus enables efficient gene delivery into both dividing and non-dividing cells. Then the cells were cultured under reprogramming conditions which are the same as for iPS cells generation. However, the reprogramming time was set as 4 days which is much shorter than the time for full iPS generation: 21-28 days (Takahashi, Tanabe et al. 2007). After 4 days of reprogramming, partially reprogrammed SMCs (PR-SMCs) exhibited morphology changes and large-scale gene expression changes. PR-SMCs started to lose their SMC identity reflected by the downregulation of a group of SMC markers. Transcriptome sequencing analysis showed that many genes had been upregulated when compared to the original SMC population, which suggested that many suppressed genes had been re-activated and the plasticity of cell identity had been triggered after reprogramming. The gene ontology analysis of molecular function indicated that genes related to the regulation of structural molecule activity were upregulated, which explains the morphological changes and the erasure of SMC signature structures. Regarding biological processes, expression of genes associated with translational processes was significantly increased.

It came as no surprise that short term reprogramming with *OCT4*, *SOX2*, *KLF4*, and *c-MYC* could fundamentally change the cellular state of SMCs. In time course studies for iPS cell generation, both genome-wide and proteome-wide analyses

revealed extensive changes at the gene and protein levels soon after the ectopic introduction of reprogramming factors, especially during the early phase from day 0 to day 3 (Buganim, Faddah et al. 2013). Other studies have used the four transcription factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* to reprogramme fibroblasts in 4-8 days to generate partially-iPS cells, which can be further differentiated towards EC or SMC in response to different stimulations (Margariti, Winkler et al. 2012, Karamariti, Margariti et al. 2013, Kurian, Sancho-Martinez et al. 2013). These studies also showed that the expression patterns of a broad range of genes related to developmental cellular activities are changed after 4-8 days of reprogramming. This supports the idea that cell identity can be significantly altered in a short time frame upon the ectopic expression of the reprogramming factors, which explains what we have observed in the 4 day-reprogrammed SMCs.

A recent study revealed that during iPS cell generation, the reprogramming factors bind to the genes that are not occupied by these factors in ESCs (Soufi, Donahue et al. 2012). This interesting fact indicates that the reprogramming factors may function in a cell type-dependent way. When specifically considering the reprogramming towards vascular cell lineage, one of the iPS-inducing reprogramming factors, *KLF4*, is particularly significant. *KLF4* has been shown to play a crucial role in regulating vascular cell development and activities in addition to its reprogramming role and has been identified as an important transcription factor for both SMC and EC. *KLF4* can inhibit the expression of SMC markers through directly binding to the TGF- β control element in their promoters or through preventing the interaction of serum response factor (SRF) with their promoters (Adam, Regan et al. 2000, Liu, Sinha et al. 2003, Liu, Sinha et al. 2005). *KLF4* has been shown to directly bind to the promoter of VE-Cadherin in mature ECs to improve cell barrier function (Cowan, Kohler et al. 2010). Another study indicated that *KLF4* plays a protective role in regulating the response of EC to inflammatory stimuli. Overexpressing *KLF4* in ECs increases the expression of anti-inflammatory and anti-thrombotic factors including eNOS and thrombomodulin (Hamik, Lin et al. 2007). In c-Kit-positive vascular progenitor population derived from ESCs, *Klf4* was shown to positively regulate their differentiation towards ECs. Overexpression of *Klf4* in this population led to further upregulation of EC markers and knockdown of *Klf4* resulted in an increase in SMC markers (Campagnolo, Tsai et al. 2015). The above studies suggest that *KLF4*

may play an additional favourable role in promoting SMC to EC conversion by suppressing SMC characteristics and possibly promoting EC identity.

To further examine the PR-SMCs population, an upregulation of the expression of CD34 was observed after 4 days of reprogramming. CD34 has been identified as an important marker for vascular progenitor cells which have the potential to give rise to both EC and SMC lineage in response to different conditions (Ferreira, Gerecht et al. 2007). It is noteworthy that CD34 has also been used as a marker for circulating endothelial progenitor cells with the capacity to facilitate neovascularisation (Mackie and Losordo 2011, Fadini, Losordo et al. 2012). The emergence of a CD34-positive population after our first step of reprogramming suggested the generation of a vascular progenitor state from SMCs. Interestingly, previous studies showed that the same set of reprogramming factors or *Oct4* alone could induce an upregulation of CD34 in fibroblasts in different culture conditions (Szabo, Rampalli et al. 2010, Kurian, Sancho-Martinez et al. 2013).

Taken together, it appears that the short-term reprogramming step facilitates the erasure of the SMC signature and drives the SMCs back to a progenitor state marked by CD34 expression. The epigenetic plasticity of this progenitor stage permits the cells to be more actively responsive to certain lineage-promoting stimuli and commit into other cell type. A recent study overexpressed *OCT4*, *SOX2*, *KLF4*, and *c-MYC* for 8 days, which was doubled reprogramming time compared to our protocol, to induce a CD34-positive mesoderm progenitor state from fibroblasts for further differentiation towards endothelial lineage (Kurian, Sancho-Martinez et al. 2013). We stopped the reprogramming at day 4 because at this time point there was already altered cell state with the CD34 upregulation and longer reprogramming time with the four factors might raise the tumourigenesis risk. However, it is still of interest to further evaluate whether longer reprogramming time can significantly increase the efficiency of the induction of CD34-positive vascular progenitors without tumour formation.

4.2.3 Differentiation of Vascular Progenitor State PR-SMCs towards an Endothelial Lineage

Following reprogramming, PR-SMCs were subjected to endothelial-promoting conditions. We chose the combination of Collagen IV coating, EGM-2 media and VEGF supplementation to create an endothelial-favouring circumstance, which is based on the established protocols that have been used to differentiate progenitors or pluripotent stem cells towards the endothelial lineage (Ferreira, Gerecht et al. 2007, Benedito, Rocha et al. 2012).

Collagen IV is an important type of ECM substrate synthesised by ECs which supports the *in vitro* differentiation of pluripotent cells towards mesoderm lineage. Collagen IV promotes the expression of mesodermal genes of endothelial and haematopoietic lineages from ESCs (Schenke-Layland, Angelis et al. 2007) and many protocols use Collagen IV to facilitate the differentiation of pluripotent/progenitor cells towards the endothelial lineage. CD34- or VEGFR2-positive cells from mouse or human pluripotent cells gave rise to ECs when cultured on Collagen IV in VEGF supplemented differentiation media (Nishikawa, Nishikawa et al. 1998, Yamashita, Itoh et al. 2000, Blancas, Lauer et al. 2008).

VEGF (more precisely VEGFA, but usually referred to just as VEGF) is a member of the secreted polypeptides VEGF family, which plays a prominent role in endothelial development and the organisation of the vasculature. *Vegf* gene knockout, even the heterozygous (+/-) knockout, causes early embryonic lethality of mice due to severe failures in vascular development (Carmeliet, Ferreira et al. 1996, Ferrara, Carver-Moore et al. 1996). VEGF mainly act through binding to VEGF receptors belonging to the receptor tyrosine kinase (RTK) family on the cell membrane: VEGFR1 (FLT1), VEGFR2 (KDR). VEGF receptors are activated through phosphorylation and carry out the effect of VEGF stimulation through several downstream signalling pathways including the PI3K/AKT pathway, MAPK/ERK pathway, etc.

Due to its important physiological role in endothelial development, VEGF has commonly been added as a potent supplement to the culture media to induce

endothelial differentiation from multiple types of stem cells. VEGF can be further classified into different isoforms with different diffusibilities and receptor binding ability resulting from the alternative splicing of the VEGF gene from exons 5 to 8(Tammela, Enholm et al. 2005). VEGF₁₆₅ that has been used in this study is the most abundant and biologically active isoform with intermediate diffusion and matrix-binding properties, which is necessary for normal vascular development (Nagy, Dvorak et al. 2003).

After 6 days of endothelial differentiation, a portion of the total PR-SMCs population was converted to endothelial-like cells with morphological changes and the expression of endothelial markers at the mRNA and protein levels. These results support the finding that the presented protocol can convert SMCs towards an endothelial lineage. However, not all of the SMCs had been switched to an endothelial fate. The flow cytometry results indicated that the upregulation of PECAM-1 expression was around 7%. The more likely explanation for this is that not every SMC responded in exactly the same way to the short time reprogramming with *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. Studies of iPS cell generation process indicate that cells respond to reprogramming factors in a highly stochastic manner. Reprogramming factors initiate a sequence of probabilistic events that eventually result in a small and unpredictable portion of cells turning into pluripotent cells (Hanna, Saha et al. 2009, Buganim, Faddah et al. 2012). Comparably, the four reprogramming factors might trigger different reactions in individual SMCs stochastically which results in only a fraction of the whole population being converted towards an endothelial lineage.

The practicability of the SMC to EC conversion protocol was further validated through use of an alternative gene delivery method to overexpress *OCT4*, *SOX2*, *KLF4*, and *c-MYC* in SMCs. SMCs transfected with a plasmid (pCAG-OSKM) that encoded the four reprogramming factors could also be steered towards the endothelial lineage through a CD34-positive intermediate vascular progenitor state. This alternative gene delivery method verifies that the four reprogramming factors are the main force to start the conversion of SMCs to vascular progenitors and further on to ECs. Another interesting finding here was that the overexpression levels of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* were increased by between 10 and 25 fold when using the plasmid for gene delivery, which was much lower than using the

lentivirus (TetO-OSKM) which could achieve changes in hundreds of fold. This difference of gene delivery efficiency can be explained by that lentivirus is a better vector to accommodate larger transgenes and the electroporation method to deliver plasmids causes certain level of cell mortality. However, the upregulation of CD34 was around 2 to 3 fold in PR-SMCs generated by pCAG-OSKM which was not significantly lower than the 4 to 5 fold CD34 induction through the lentiviral method. Therefore, the absolute expression level of reprogramming factors might not be a key issue for the efficiency of SMC to EC conversion. As previously suggested, it is possible that only a fraction of SMCs responded to stimulation with the reprogramming factors. Single cell tracing and analysis should be further performed to confirm this hypothesis.

4.2.4 Selection of CD34-positive cells in PR-SMCs Displays the Ability to Differentiate towards a More Homogenous Endothelial Population

To further improve the efficiency of the established protocol, CD34-positive cells were selected from the whole PR-SMCs population to reduce the negative effect caused by the heterogeneity of the cell population. CD34 selection has been applied in many endothelial differentiation protocols in order to generate a homogenous mesodermal vascular progenitor or endothelial progenitor population for subsequent differentiation towards ECs. For example, a recent study reprogrammed fibroblasts with *OCT4*, *SOX2*, *KLF4*, and *c-MYC* and then used CD34 as the marker to select mesoderm precursors for differentiation to ECs (Kurian, Sancho-Martinez et al. 2013).

Indeed, CD34-positive SMC-derived vascular progenitors exhibit an enhanced capacity to give rise to a more homogeneous endothelial population with a complete repertoire of endothelial features compared to the heterogeneous vascular progenitor population. PECAM-1 and VE-Cadherin expression levels had been enhanced to around 30% based on the flow cytometry analysis. The establishment of endothelial junctions formed by adhesive proteins and the signals transferred by them are

important indicators for successful EC generation. Expression of the typical endothelial junction markers PECAM-1, VE-Cadherin, and Claudin5 was detected at the mRNA and protein levels in SMC-CD34-ECs. In addition, gene ontology analysis for all the upregulated genes in SMC-CD34-ECs revealed genes that related to the biological processes of cell adhesion and cell surface receptor linked signal transduction were highly induced. Taken together, these data suggest a profile which supports the formation of endothelial cell-cell contacts. Gene ontology analysis of the molecular functions showed that genes related to calcium ion binding and carbohydrates binding were upregulated in SMC-CD34-ECs. Cytoplasmic calcium participates in regulating many aspects of endothelial function, including the synthesis and release of NO, signal transduction by membrane proteins, platelet adhesion, etc (Tran and Watanabe 2006). Selectins are one example of important EC adhesion molecules which are calcium-dependent and contain carbohydrate binding domains for interaction with specific glycans on platelets and leukocytes (Somers, Tang et al. 2000). The upregulation of genes related to calcium ion binding and carbohydrate binding therefore suggests the activation of endothelial function in SMC-derived ECs.

The strongest evidence for efficiency of the protocol is that the SMC-derived ECs exhibit endothelial related functions *in vitro* and *in vivo*. In addition to the observation that endothelial marker eNOS was strongly upregulated during SMC to EC conversion, SMC-derived ECs were also shown to have acquired NO producing capacity, which is an important feature of functional ECs. SMC-CD34-ECs could efficiently take up acetylated-LDL which demonstrated that their lipid metabolic ability resembled normal ECs. *In vitro* and *in vivo* angiogenic capacity of the SMC-derived ECs was assessed using an *in vitro* Matrigel tube formation assay and an *in vivo* Matrigel plug assay. SMC-CD34-ECs showed the ability to assemble capillary-like structures *in vitro* and *in vivo* which indicated the EC-resembling angiogenic function.

4.2.5 Summary of the Established Protocol

Taken together, we presented here a short and efficient protocol allowing the conversion of SMCs into functional ECs (Figure 4.1). Compared with existing protocols to generate ECs from stem cells or other types of somatic cells, our protocol has a clear advantage in that it lasts only 10 to 15 days, which is time-efficient compared to existing protocols. We have also used a novel cell type, SMCs, to achieve the generation of functional ECs expressing a full repertoire of EC markers. Compared to ESCs or iPS cells as cell sources for functional EC generation, SMC can be obtained autologously without any ethical concerns. The tumour formation also was not observed during the whole study. The finding that SMCs can be used as a potential new cell source for ECs brings interesting physiological, pathological and therapeutic implications. It is possible to develop a therapeutic strategy to directly convert native SMCs to repair damaged endothelium *in vivo*. These will be further discussed in Chapter 4.4.

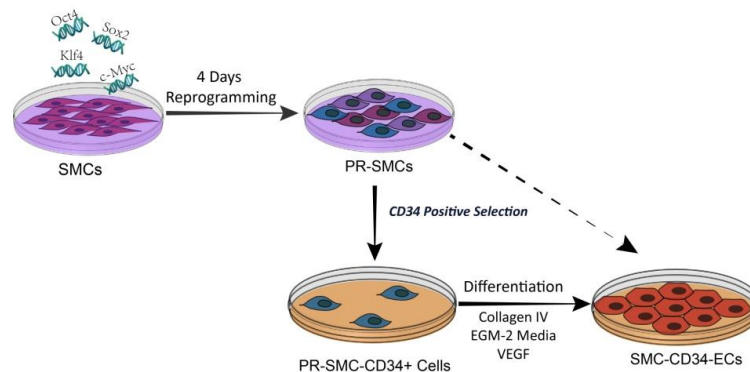


Figure 4.1 Schematic overview of the protocol to derive ECs from SMCs

The derivation of ECs from the SMCs is achieved based on a combined protocol of reprogramming SMCs to CD34-positive progenitor and subsequent differentiation towards an endothelial lineage. SMCs are reprogrammed with four transcription factors for 4 days to generate PR-SMCs. PR-SMCs are partially able to differentiate towards an endothelial lineage. To promote the efficiency, CD34-positive cells are selected from the PR-SMCs which can give rise to a more homogenous endothelial population.

4.3 Mechanisms Involved in the Conversion of SMC towards EC

4.3.1 The Involvement of MET and E-Cadherin in the Induction of a Vascular Progenitor State from SMCs

Accumulating evidence has shown that MET is a vital event that must occur at the initial stages of reprogramming for successful iPS cell generation (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010, Hansson, Rafiee et al. 2012). Reprogramming factors coordinate a comprehensive molecular network to drive the occurrence of MET and the reversion of differentiated cells back to pluripotency. *OCT4*, *SOX2*, *KLF4*, and *c-MYC* have been indicated to regulate MET through several different mechanisms. In this study, we used the four reprogramming factors to induce the vascular progenitor state from SMCs. SMCs can be considered as mesenchymal-like cells due to their lack of intercellular adhesion/junction contacts and their ability to migrate through ECM. During the 4 days of reprogramming from SMCs to vascular progenitors, SMCs gradually lost their mesenchymal-like morphology. Simultaneously, there was an overall downregulation in mesenchymal markers and EMT-promoting transcription factors, corresponding with an overall upregulation in cell-cell adhesion markers, especially E-Cadherin, expression of which is a characteristic event of MET. The occurrence of MET, induced by the four reprogramming factors, indicated the modulation of SMC plasticity and identity during the 4 day reprogramming.

E-Cadherin expression, a crucial indicator of MET, may have an additional role in regulating the generation of the vascular progenitor state from SMCs, which is possibly through modulation of the establishment of cell-cell contacts. Knockdown of E-Cadherin during 4 day reprogramming disrupted CD34-positive population induction from SMCs and abolished further endothelial differentiation. E-Cadherin has been shown to play an important role in establishing proper cell-cell contacts during iPS cell generation (Chen, Yuan et al. 2010). An early study suggested a role of E-Cadherin in adhesion of a CD34-positive subset of human bone marrow stromal

cells (Turel and Rao 1998). In the context of SMC to EC conversion, cell junction proteins including CD34 and many important endothelial markers such as VE-Cadherin, PECAM-1, and Claudin 5 are induced. The accurate establishment of contacts between neighbouring cells mediated by E-Cadherin may be a requisite event for the further establishment of vascular progenitor and endothelial junction proteins.

4.3.2 Components of the Notch Signalling Pathway Participate in Endothelial Differentiation from a Vascular Progenitor State

The Notch signalling pathway participates in regulation of diverse vascular cell activities during embryonic and postnatal development (Bray 2006). The Notch signals usually function to drive the decisions of the precursors between two alternative fates (Artavanis-Tsakonas and Muskavitch 2010). For example, Notch signals determine the arterial-venous fate of ECs and the tip or stalk cells selection of ECs during angiogenesis (reviewed in Chapter 1.1.4.4). Comprehensive analysis of RNA-Seq data demonstrated the likely involvement of several Notch components in SMC to EC conversion: JAG1, DLL4, HES5, and HEY2. To further confirm which Notch pathway members participate in endothelial induction, real-time PCR was performed to check these four candidates as well as several other Notch components that have been reported to be important for the regulation of endothelial development or function: NOTCH1, NOTCH4, HES1, and HEY1. During endothelial induction from the vascular progenitor state PR-SMCs, HES5 and JAG1 showed increased expression at the transcriptome and mRNA levels. The activation of HES5 and JAG1 expression at the protein level was further confirmed. Therefore, our interests expanded to exploring the roles of HES5 and JAG1 in regulating vascular progenitor PR-SMC to endothelial differentiation.

4.3.2.1 The Involvement of HES5 in Endothelial Differentiation

HES5 is a common downstream target of the Notch pathway which belongs to the basic helix-loop-helix transcription factor family and is usually associated with neural cell differentiation (Iso, Kedes et al. 2003). In our protocol, HES5 was upregulated in concert with EC markers induction. Moreover, by knocking down or overexpressing HES5 during endothelial differentiation, we observed a repression or increase in EC marker expression respectively. These results indicated the positive regulatory function of HES5 during endothelial induction. HES5 has not been related to endothelial development or function in many published works. One study suggested a role for HES5 in vascular development, as it might be a key positive mediator for the statin-induced differentiation of bone marrow stromal cells into ECs (Xu, Liu et al. 2009). Another study has suggested that HES5 plays a part in promoting endothelial proliferation in response to endothelial injury during atherosclerosis (Schober, Nazari-Jahantigh et al. 2014). This study also demonstrated that MicroRNA-126-5p promotes endothelial regeneration and limits atherosclerosis by suppressing the Notch inhibitor delta-like 1 homologue (Dlk1), which leads to release of HES5 that has been suppressed by Dlk1 to play its role in endothelial repair.

It is worth pointing out that the endothelial stimulation role of HES5 could only promote endothelial differentiation in the vascular progenitor population derived from SMCs after induction by the four reprogramming factors, whereas HES5 hardly triggered EC marker expression on its own when overexpressed in the control cell population. All the findings above imply that the four reprogramming factors open up certain opportunities for HES5's further regulation of endothelial related gene expression. Using a mouse iPS cell to EC differentiation model, the upregulation of Hes5 had been observed along with the induction of endothelial markers. This result indicated that the endothelial-regulatory role of Hes5 may not only be restricted to the SMC to EC conversion.

HES5 has usually been described as a transcription repressor, however in some cases it can also function as transcription activator (Iso, Kedes et al. 2003, Kamakura, Oishi et al. 2004). Our data suggest that HES5 might play a part in activating the eNOS promoter. Additional studies will be required to elucidate a more detailed

mechanism which could be HES5 directly binding to the promoter region or activating it through other factors. There have been reports about HES5 enhancement of the phosphorylation of JAK2, which plays an important role in eNOS activation (Cieslik, Abrams et al. 2001, Kamakura, Oishi et al. 2004).

In addition to the promotion of EC profile induction, overexpression of HES5 led to the simultaneous further repression of SMC marker. HES5 has been reported to repress downstream transcriptional enhancers such as Mash (de la Pompa, Wakeham et al. 1997), MyoD (Kopan, Nye et al. 1994) and Myocardin (Proweller, Pear et al. 2005), which are all related to the positive regulation of SMC marker promoters. Using pulmonary arterial hypertension (PAH) as a pathological model to study the plasticity and proliferation of vascular SMCs, Li *et al.* showed that HES5 participated in regulation of arterial SMC proliferative capacity and differentiation. HES5, corresponding with NOTCH3, suppressed the contractile marker expression of vascular SMCs including SM-MHC and Smoothelin in response to hypoxia-induced PAH (Li, Zhang et al. 2009). Therefore, HES5 might act to suppress the SMC transcription network to repress the SMC differentiation fate of PR-SMC and promote the endothelial differentiation fate.

4.3.2.2 The Involvement of JAG1 in Endothelial Differentiation

JAG1 is a Serrate/Jagged family transmembrane ligand for the Notch pathway containing multiple epidermal growth factor-like repeats (Fleming 1998). JAG1, as the upstream ligand of the transmembrane receptors in the Notch pathway, plays a complicated role in orchestrating cell fate. The homozygous mutation of the *Jag1* gene in mice causes early embryonic lethality due to extensive embryonic and yolk sac vascular defects (Xue, Gao et al. 1999). The mutation of the *JAG1* gene in humans leads to Alagille syndrome characterised by abnormal development of multiple systems during childhood (Li, Krantz et al. 1997, Oda, Elkahloun et al. 1997). Vascular anomalies including pulmonary artery abnormalities, intracranial haemorrhages and other events, frequently occur to Alagille syndrome patients and account for a large portion of mortality, which reflects the important role of *JAG1* during human vascular development (Kamath, Spinner et al. 2004).

The pro-angiogenic role of Jag1 has been shown using EC-specific and inducible knockout or overexpression in mice. *Jag1* loss-of-function mutants exhibited reduced sprouting angiogenesis while *Jag1* overexpression promotes sprouting angiogenesis (Benedito, Roca et al. 2009). Kwon *et al.* showed that *Jag1*-induced signals from the bone marrow microenvironment are critical for the development of angiogenic ability of endothelial progenitor cells (Kwon, Eguchi et al. 2008). An interesting study recently demonstrated that JAG1 could subsequently activate KLF4 which induced the transdifferentiation of tumour cells into endothelial cells (Chen, Huang et al. 2014).

The above studies provide evidence for JAG1 facilitating endothelial differentiation and angiogenesis. Nevertheless, there are reports of the opposite effect in other cell models. A recent study emphasised the role of JAG1 in promoting haematopoietic lineage over endothelial lineage specification during pluripotent stem cell differentiation (Lee, Werbowetski-Ogilvie et al. 2013). It is conceivable that in different cell types and in response to different environmental cues, the JAG1 activated Notch pathway may delicately control a distinct regulation network which leads to altered consequences.

In this study, we revealed that JAG1 participates in the regulation of the expression of some EC markers especially eNOS, during vascular progenitor to endothelial lineage differentiation. We detected that upon JAG1 stimulation, eNOS was upregulated at the mRNA level through the activation of its promoter. This finding coincided with a recent report which showed that JAG1 activation in response to VEGF stimulation is required for eNOS activation and NO production in ECs (Patenaude, Fuller et al. 2014). Furthermore, we showed that the HES5 promoter could be activated upon JAG1 overexpression. Therefore, it is possible that the endothelial promoting role of JAG1 was carried out through HES5.

4.4 Potential Applications of SMC-derived ECs

Cell-based therapeutic angiogenesis is a recently arising approach to restore blood perfusion to ischaemic tissue. Successful therapeutic angiogenesis depends on the transplanted cells to directly incorporate into the neovasculature as well as to secrete angiogenic growth factors (Raval and Losordo 2013). This therapy is of special significance in treating peripheral artery disease (PAD) since current pharmacological and interventional revascularisation therapies are not beneficial enough (Annex 2013). However, researchers are still investigating the optimal starting cells to generate functional angiogenic cells. In a murine ischaemia model of PAD for this study, we demonstrated that SMC-derived ECs could efficiently engraft into local vasculogenesis of ischaemic tissue and profoundly improve the tissue perfusion. Moreover, to directly convert SMCs to ECs without reversing to a pluripotent state prevents the risk of tumour formation. Our results indicate that SMC-derived ECs could be a promising candidate for vascular regenerative therapies.

Tissue engineered vascular graft represents another promising direction for vascular regenerative medicine. In addition to direct transplantation to replace injured vessels, tissue engineered graft can also serve as a useful *ex vivo* model to study the mechanisms related to vascular cell or ECM behaviours. Based on a previously established protocol from our laboratory, functional vascular-resembling conduits can be generated from seeding the decellularised mouse aorta with human origin cells using an *ex vivo* bioreactor circulation system (Karamariti, Margariti et al. 2013, Wong, Hong et al. 2015). Adopting this model in our study, seeding the lumen of the decellularised vascular grafts with SMC-derived ECs led to successful re-endothelialisation which suggested their potential implications in constructing tissue engineered vascular graft. The evaluation of the *in vivo* function of the reconstructed vascular graft needs to be further performed.

Another important implication of SMC-derived ECs is in the context of restenosis. Vascular intervention therapies such as balloon angioplasty or endovascular stent implantation provide beneficial solutions to patients suffering from narrowed vessels. However, one of the main concerns of intervention therapies is the further damage to the endothelium which can occur during the procedure which triggers restenosis, the

proliferation and migrating of SMCs into the intima and finally the re-narrowing of the vessel (Chaabane, Otsuka et al. 2013). Certain drugs like sirolimus, paclitaxel, everolimus have been applied with intervention therapies to inhibit SMCs proliferation and suppress neointima formation to prevent restenosis. The application of these drugs with intervention therapies significantly reduces the clinical incidence of restenosis (De Luca, Dirksen et al. 2012). However, these drugs indiscriminately inhibit the proliferation and migration of ECs at the same time, which further impair and delay the endothelial repair. It is of great interest to explore the endothelial regeneration strategy for restenosis.

The proliferation and accumulation of SMCs following endothelial denudation/dysfunction profoundly contributes to the development of atherosclerosis and restenosis. Our protocol for the direct conversion from SMCs into functional ECs may provide a promising tool for *in situ* endothelial regeneration. However, the main obstacle to this future application is the lack of proper gene delivery technique aiming at the specific type of cell for *in vivo* reprogramming. A recent study demonstrated an *in situ* virus delivery method to specifically target vascular SMCs without effecting ECs by constructing a designated gene with EC enriched microRNA target sequences within the same vector (Santulli, Wronska et al. 2014). By employing a similar strategy, it is possible to develop a method to specifically switch local SMCs into the endothelial lineage to achieve autologous endothelium repair.

The fast development in the field of bioengineering and biomaterial research provides new ideas for precise *in vivo* gene delivery. For example, a promising bioengineering technique for gene delivery is microfabricated microneedles. Microneedle techniques had originally been used for intra- and trans-cutaneous delivery of genes or drugs through transdermal tissue penetration (McAllister, Allen et al. 2000). A perivascular microneedle cuff has been developed to deliver genes or drugs to the tunica media layer of vascular wall (Lee, Park et al. 2014). A gene delivery strategy combination of SMC-target expressing vector and microneedle delivery system may provide a potential solution for *in vivo* conversion of SMCs to ECs and re-endothelialisation.

In conclusion, the feasibility of converting human SMCs into functional ECs provides novel application possibilities for treating cardiovascular diseases.

4.5 Summary and Perspective

In summary, this is the first study to show that functional ECs can be efficiently derived from SMCs through an intermediate vascular progenitor state which is induced by reprogramming factors *OCT4*, *SOX2*, *KLF4* and *c-MYC*. We also provided insights into the mechanisms behind the process involving MET and the Notch signalling. However, there are limitations to the present study and further work need to be conducted.

First, to use reprogramming factors, especially the oncogene *c-MYC*, as part of the protocol still raises tumourigenesis concerns. Although teratoma formation has not been observed in any *in vivo* experiments of this study, the long term effect of using reprogramming factors is difficult to predict. In addition, using lentivirus as the vector to deliver genes also has potential safety concerns. The technique of reprogramming has been rapidly developing since the design of this study. In addition to transcription factors, many other kinds of small molecules and chemical defined conditions have been applied to conduct reprogramming including microRNAs, epigenetic regulators, and signalling pathway regulators. For example, a recent study used a toll-like receptor agonist to successfully reprogramme fibroblasts into ECs (Sayed, Wong et al. 2015). Therefore, it is of great interest to explore the possibility of using small molecules to partly or fully replace reprogramming factors to achieve the direct conversion of SMCs towards ECs, which can reduce the safety concerns for using pluripotency factors and the lentivirus. In addition, further optimisation of the protocol could be carried out to improve the purity and stability of the converted ECs. Moreover, an optimised culture condition for the long term maintenance of the SMC-derived ECs needs to be established.

We have shown that the SMC-derived ECs acquire EC-like characteristics and functions, especially the therapeutic angiogenic ability and bioengineered vascular graft constructing capacity. In this study, we have focused on evaluating the generation of the endothelial phenotype from SMC and the acquisition of endothelial functions. For our next step, it would be of great interest to directly compare this SMC-derived endothelial population with their native counterparts ECs and the ECs

derived from other cell sources such as ESC-ECs, iPS-ECs, and EPC-ECs at the molecular and functional levels. A detailed comparison among different cell source-derived ECs could provide further indications for choosing the suitable cell population for therapeutic purposes.

The regulatory roles of HES5 and JAG1 for endothelial differentiation have been discussed in the present study. However, further work need to be done to elucidate the exact regulatory mechanisms between HES5 and JAG1 and their precise roles on endothelial differentiation. In addition, the possibility of modulating the Notch signalling pathway to achieve SMC to EC conversion can be further explored.

One of the promising therapeutic applications of the SMC to EC conversion is in the restenosis scenario, since the over-proliferating SMCs can be a direct source for *in situ* endothelial regeneration. The SMC-derived ECs can be transplanted to test their endothelial repair ability in the wire-injured vascular restenosis model. Furthermore, the appropriate *in vivo* gene delivery method, especially to precisely target SMCs within the vascular wall, will be explored.

In conclusion, although future works are required to further complete and extend this subject, the present study has proven the hypothesis of this project that it is feasible to derive functional EC from human SMC. The aims of this study have also been achieved: (1) a protocol has been established to derived EC from human SMC through short time reprogramming and subsequent endothelial differentiation; (2) SMC-derived EC displays typical EC functions including NO production, ac-LDL uptake, *in vitro* and *in vivo* angiogenesis, and further therapeutic angiogenic capacity and the ability to construct an endothelial layer in tissue engineered vascular graft; (3) MET and components of the Notch signalling pathway have been implicated underlying different stages of the derivation of EC from SMC.

LIST OF PUBLICATIONS

Hong X, Margariti A, Jacquet L, Le Bras A, Kong W, Hu Y, Xu Q (2015): Conversion of human smooth muscle cells towards functional endothelial lineage enabling therapeutic angiogenesis, *Circulation Research*, under revision.

Simpson R, **Hong X**, Wong MM, Karamariti E, Bhaloo SI, Warren D, Kong W, Hu Y, Xu Q (2015): Hyaluronan Is Crucial for Stem Cell Differentiation into Smooth Muscle Lineage. *Stem Cells*, under revision.

Wong MM*, **Hong X***, Karamariti E, Hu Y, Xu Q (2015): Generation and Grafting of Tissue-engineered Vessels in a Mouse Model, *Journal of Visualized Experiments*, 2015 Mar, 97. (*Authors contributed equally)

Campagnolo P, Tsai T, **Hong X**, Kirton JP, Margariti A, Bernardini ED, Wong MM, Hu Y, Xu Q (2015): C-Kit+ progenitors generate vascular cells for tissue-engineered grafts through modulation of the Wnt/Klf4 pathway, *Biomaterials* 60: 53-61.

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Conference Poster Presentation

X Hong, Margariti A, Jacquet L, Le Bras A, Kong W, Hu Y, Xu Q. Generation of Functional Endothelial-like Cells Enabling Therapeutic Angiogenesis from Human Smooth Muscle Cells. British Cardiovascular Society/British Atherosclerosis Society Annual Conference. Manchester, UK. June 2015

Simpsons R, **Hong X**, Xu Q. Hyaluronan is required for Smooth Muscle Cell Differentiation from Stem Cells. British Cardiovascular Society/British Atherosclerosis Society Annual Conference. London, UK. Jun 2013

Campagnolo P, **Hong X**, Xu Q. Resveratrol Reduces Vessel-Graft Neointimal Formation by Inducing Endothelial Differentiation of Resident Progenitor Cells through MiR-21/AKT/ β Catenin Pathway. British Cardiovascular Society/British Atherosclerosis Society Annual Conference. Jun 2013

FUNDING SOURCES

This study was supported by grants from the British Heart Foundation and the Oak Foundation. I am supported by the scholarship from the China Scholarship Council.

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